

**Investigating phenotypes of asthma in
elite performance athletes.**

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Abstract

Investigating phenotypes of asthma in elite performance athletes.

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There is a high prevalence of exercise induced bronchoconstriction (EIB) in elite athletes. It has been suggested that damage to the airway epithelium is the key effector process and that EIB in athletes is due to different mechanisms than exercise induced asthma. Whether testing strategies are as valid in athletes is controversial, but regulatory bodies continue to advocate the use of objective tests for diagnosis. How responses to these relate to sport, airways inflammation and to symptoms is unclear. We investigated responses to direct and indirect challenge tests and patterns of airways inflammation in symptomatic, international, endurance athletes. We focused on differences between pool-based and non-pool based endurance athletes to see if environmental factors played a significant role. We also investigated the interaction between airways epithelial and human lung mast cells *in vitro* and compared these between healthy and asthmatic donors.

Of the challenges assessed, EVH related most closely to eosinophilic airways inflammation. The other tests examined did not relate particularly closely to each other, to eosinophilic airways inflammation or to markers of mast cell activation. There were no differences between pool and non-pool based athletes in terms of patterns of airways inflammation or airway mediator release in response to challenge testing. Pool-based athletes had significantly more airways hyperactivity when compared to non-pool based athletes. Those who test positive to EVH have more eosinophilic airways inflammation and more epithelial cells in their sputum than those who have a negative test. All indirect challenge tests increased the level of PGE₂ in the airways compared to direct testing, even when corrected for degree of bronchoconstriction, suggesting epithelial stress. *In vitro*, an intact, healthy epithelium significantly suppresses constitutive and IgE mediated human lung mast cell histamine secretion. This suppression is attenuated in asthmatic or injured epithelium and is mediated by a small, labile, lipid soluble mediator.

There is a significant heterogeneity in airways inflammation and airways hyperactivity in elite performance athletes. The role of the epithelial cell in the development of EIB requires further exploration. The interaction between the epithelium and human lung mast cells needs to be fully elucidated and its potential for therapeutic manipulation further explored. (355)

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Finally, as I write this sitting in the Role 3 Hospital in Camp Bastion, Afghanistan, I am daily reminded why we choose to practice medicine and why clinical research is so important. I hope that some of the science we have used here will have an impact in disease treatment or prevention in the future and so directly benefit the patients that we see in daily practice.

Publications list

Martin N et al

Association of elevated nitric oxide and eosinophilic airways disease in elite athletes with exercise induced bronchoconstriction
ERS Conference Thematic Poster Discussion, Vienna 2009

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UKSEM, London, Nov 2010.

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Epithelial suppression of constitutive mast cell function in health and asthma.
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Abbreviations

ACQ	Asthma control questionnaire
AEC	Airways epithelial cells
AHR	Airways hyper-responsiveness
ALI	Air liquid interface
ANOVA	Analysis of variance
AQLQ	Asthma quality of life questionnaire
ASM	Airways smooth muscle
CI	Confidence intervals
COX-1	Cyclo-oxygenase 1
COX-2	Cyclo-oxygenase 2
CYST-LT	Cysteinyl leukotrienes
EIA	Exercise induced asthma
EIB	Exercise induced bronchoconstriction
EVH	Eucapnic voluntary hyperventilation
FEV ₁	Forced expiratory volume in one second
FE _{NO}	Fraction of exhaled nitric oxide
FVC	Forced vital capacity
HDM	House dust mites
HLMC	Human lung mast cells
IOC-MC	International Olympic Committee – Medical Committee
LTC ₄	Leukotriene C ₄
Mann PD15	Mannitol provocative dose for a 15% fall in FEV ₁
Meth PC20	Methacholine provocative concentration for 20% drop in FEV ₁
PGD ₂	Prostaglandin D ₂
PGE ₂	Prostaglandin E ₂
PGF ₂	Prostaglandin F ₂
PTX	Pertussis toxin
SD	Standard deviation
SEM	Standard error of the mean
UHL	University Hospitals of Leicester
WADA	World anti-doping agency

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1.0 Introduction

1.1 Asthma

Asthma is a condition that is characterised by variable airflow obstruction and airway hyper-responsiveness in association with airways inflammation, that usually has an eosinophilic component (1). Pathologically, together with the accumulation of eosinophils there is an increase in lymphocytes, dendritic cells and macrophages with activation of mast cells and epithelial cells (2). Inflammatory mediators secreted by these and other cells contribute to changes in airway function and structure. The structural changes include thickening of the sub-epithelial collagen layer and submucosal matrix deposition (3), hyperplasia and hypertrophy of goblet cells with mucus hypersecretion (4) and smooth muscle hypertrophy and hyperplasia (5). These structural changes form the basis of the ‘airway remodelling’ that is another characteristic of asthma. In addition epithelial desquamation, or at least fragility, has been considered an important hallmark of the disease (6).

Asthma remains a clinical diagnosis and central to all definitions is the presence of symptoms (more than one of wheeze, breathlessness, chest tightness, cough) and of variable airflow obstruction (7). More recent descriptions of asthma in children and in adults have included airway hyper-responsiveness and airway inflammation as components of the disease but how these features relate to each other, how they are best measured and how they contribute to the clinical manifestations of asthma, remains unclear (8). What is certain is that there is marked heterogeneity in airways inflammation and hyperreactivity for often very similar clinical presenting features . At the severe end of the asthma spectrum this has led to great interest in characterizing patients in greater detail looking for detailed disease phenotypes (9) as a means to explore their response to traditional and emerging therapeutic options. Key to the determination of these phenotypes has been the determination of the presence or absence of

eosinophilic airways inflammation and its inherent steroid responsiveness (10). This has facilitated improved management of severe asthma and permitted the development of more advanced treatments (11). However, how these phenotypes translate to patients at the less severe end of the spectrum or to particular specialized groups remains to be fully explored and developed.

One of the most common and pervasive symptoms of asthma is breathlessness and wheeze on or shortly after the commencement of exercise (12). Traditionally this has been felt to be due to poor underlying asthma control and symptoms usually show a marked improvement with the addition of further therapy (7). However, in some patients this remains a significant factor in their daily asthma morbidity and in some specialized groups of patients this increased need for medication may have significant occupational consequences (13;14). Little is known about the phenotypes of disease in these often young, fit and otherwise healthy individuals. A simple guideline directed approach to their management may not necessarily be appropriate and a clinical based diagnostic process may simply not be sufficiently robust (15). Whilst these otherwise healthy individuals are likely to be low users of healthcare services there is the potential for significant impact of both the diagnosis and the management of the disease on their work and lifestyle that is largely ignored in modern guideline directed approaches to disease management (16). One such group is elite performance athletes.

1.2 Asthma in athletes.

There is a high prevalence of asthma amongst elite performance athletes but how best to assess, diagnose and treat asthma in athletes remains controversial. The underlying high cardiorespiratory fitness levels of athletes make the diagnostic process more complex as do a variety of both common and rare alternative diagnoses that must be considered. To add to this

the pathophysiology of exercise induced bronchoconstriction in athletes is believed to differ significantly from the exercise induced asthma seen in clinical patients, with less allergic inflammation and less steroid responsive airways pathology. They are a highly specialised group of patients that require detailed testing to use standard medication during competition, yet we know little of the underlying inflammatory phenotypes within this group.

1.2.1 Prevalence of asthma in elite athletes

The prevalence of asthma in the elite sporting population far outstrips that in the general population, particularly in endurance athletes and especially in those who train in an environment with further provoking factors such as cold air (17), chemical products in pools (18), and a combination of these in indoor ice rinks (19). The prevalence may be increasing as indicated by the fact that the number of athletes using medication in elite level sport has been increasing steadily over recent years (20). Early questionnaire based studies estimated the prevalence of symptoms suggesting exercise asthma to be between 11-30 % in summer athletes and up to 50% in winter athletes and swimmers (21) against a background population prevalence in adults of about 15% (22) (table 1). However, symptom responses on questionnaires alone likely provide a limited perspective (23) as Rundell et al showed that there is poor agreement between symptoms and objective measures of airway dysfunction (24). In this study, half of athletes reporting symptoms on questionnaire had negative airway challenge tests and half of those with positive challenge tests reported no symptoms or functional limitation. A possible explanation for this discrepancy is that improvement in cardiovascular and musculoskeletal training techniques allow athletes to function at the limit of their respiratory capacity so that it is difficult to discriminate between physiological and pathological limitations to maximum performance. Despite this caveat, cross-sectional studies have consistently shown an increased prevalence of abnormal objective tests of

airway dysfunction and inflammation in athletes. A summary of these studies is presented in Table 1.1.

Since the 2002 Salt Lake City winter Olympic games the Medical Committee of the International Olympic Association (IOC-MC) has required the submission of objective tests of airways dysfunction before athletes are allowed to use medication in elite level sports (20). This has not appreciably altered the numbers of athletes who compete with a diagnosis of exercise asthma but there has been a reduction in some sports and from certain countries (25). This approach has allowed for an improvement in diagnostic accuracy and has encouraged more directed and focused research.

1.2.2 Pathophysiology of exercise induced bronchoconstriction

Exercise-induced bronchoconstriction (EIB) is triggered by the inhalation of cold or dry air in approximately 50% of asthmatic patients (26) and 10-20% of all adolescents irrespective of asthma diagnosis (27;28). The pathophysiology of these exercise related symptoms is distinct but complex, involving the release of eicosanoids, cysteinyl-leukotrienes and prostaglandin-D₂ into the airways from mast cells, eosinophils and other airway cells. However, the mechanism by which the release of these products into the airways in response to exercise challenge leads to airflow obstruction is incompletely understood (29). There are two competing hypotheses: loss of heat leading to vascular engorgement as the airways re-warm after exercise, mediator release and bronchoconstriction (30); and loss of water leading to a change in the airway lining fluid osmolarity, epithelial and mast cell activation, and secretion of inflammatory bronchoconstrictor mediators (31).

There is compelling evidence that mast cells play a key role in exercise induced asthma. Bronchoconstriction can be attenuated by mast cell inhibition (32) and by histamine and cysteinyl-leukotriene antagonists (33), and the levels of prostaglandin and cysteinyl-leukotriene products are increased in the airway and in urine after exercise-induced bronchoconstriction (34). Cysteinyl-leukotrienes are particularly important in this process and modulation of the 5-lipoxygenase pathway offers a potential mechanism for attenuating airways dysfunction secondary to exercise (35). Recent evidence suggests that the location of mast cells may also play an important role in the development of airway hyperresponsiveness and perhaps exercise-induced asthma (36). Comparative pathological studies have shown increased numbers of mast cells in the airway smooth muscle of individuals with asthma irrespective of the airway mucosal pathology, and the absence of airway smooth muscle mast cells in non-asthmatic, eosinophilic bronchitis (37).

Within the general asthma population the consensus remains that the presence of exercise related symptoms reflects poor underlying control of airways inflammation (7). Traditionally this has been called exercise induced asthma (EIA). Usually other symptoms associated with asthma are present, there is objective evidence of eosinophilic inflammation and long-term use of inhaled corticosteroids results in improvement of all these features (38). The situation is different in athletes, as EIB is often experienced in the absence of other symptoms, suggesting that the mechanisms and underlying pathophysiology may be different.

Several lines of evidence support this view. Firstly, the airway inflammation in athletes has been shown to be less steroid responsive (39) and more heterogeneous than that seen in classic asthma (40;41). Secondly, within the population of athletes with exercise-induced bronchoconstriction there are marked differences in atopy between summer and winter

athletes and between different sports (42) whereas EIA is closely associated with atopy. Finally, there may be a causal relationship between training intensity and environment and increasing airway inflammation, airway remodelling and airway responsiveness in athletes (31) whereas all the evidence suggest that increasing exercise and physical fitness has beneficial effects in subjects with EIA (43;44).

The concept that EIB is caused by repeated high-level exercise is supported by studies showing that removal of swimmers from a competitive environment results in an improvement in symptoms (43). Training duration may also be a significant factor as younger athletes tend to have less airways dysfunction and inflammation compared with their older sporting equivalents (45). How might repeated exercise lead to airway dysfunction and inflammation? One possibility is that changes in the heat and water retaining properties of the airway surface layer, airway drying and the development of an osmotic gradient across the airway epithelial cells are important causal events (46). In support of this, EIB can be affected by dietary salt intake (47) and epithelial cells ability to control airway hydration (48). Moreover, it has recently been demonstrated that bronchoconstriction after exercise challenge is associated with significantly increased expression of the gel forming mucin MUC5AC (designed to protect against airways epithelial cell dehydration (49). Although this is not unique to athletes it may be that this plays a more important role in the pathophysiology of EIB in athletes and may result in less steroid responsive inflammation than occurs with atopic sensitisation.

Up to 50% of patients with EIA demonstrate a refractory period during which repeated exercise challenge induces significantly less bronchoconstriction. This lasts for 1-3 hours following exercise (50;51). A variety of theories for the development of the refractory period

have been proposed including better airway thermal protection, depletion of airway inflammatory mediators or reduction in catecholamine secretion. However, there has been no demonstrated reduction in heat and water loss (52), nor depletion or reduction in plasma histamine, neutrophil chemotactic activity or increase in plasma catecholamines during the refractory period (53). The bronchoconstrictor response to directly acting stimuli such as methacholine is unchanged during the refractory period indicating that loss of airway smooth muscle contractility is not the cause. In contrast, subjects rendered refractory to one stimulus (i.e exercise) are also refractory to other indirectly acting stimuli, even if the mechanism of bronchoconstriction is distinct (54) (i.e. inhaled sodium metabisulphite, which induces bronchoconstriction via neural pathways). These findings imply that the refractory period is due to production of an endogenous bronchoprotective factor which has a suppressive effect on pathways involved in the response to indirect bronchoconstrictor challenges. The refractory period is abolished by the COX-1 and COX-2 inhibitor indomethacin in either oral (55) or inhaled form (56) suggesting a role for a bronchoprotective prostaglandin (PG) such as PGE₂. There may be other mechanisms involved, as in vitro studies have shown that mast cell degranulation is inhibited by epithelial cells via a non-prostanoid mechanism (57). It remains to be demonstrated whether the refractory period is present to such a degree in elite performance athletes. It is possible that repetitive periods of airways drying in athletes leads to loss of this bronchoprotective mechanism because of epithelial cell injury, airways damage and the development of chronic airway inflammation (58).

There remains considerable uncertainty about the mechanism of EIB in athletes and the degree to which this differs from EIA. Interpretation of the available studies is often difficult as much of the data is derived from small populations and the findings are often inconsistent between studies. Moreover, the clinical relevance of any identified differences has not been

explored. It is perhaps most likely that exercise-induced bronchoconstriction is a heterogeneous entity with a complex relationship between endogenous factors, training intensity and training environment on one hand, and symptoms, functional limitation, airway inflammation and airway dysfunction on the other. We suggest that an approach to assessment which breaks the syndrome down to its individual component parts might be fruitful. This method has been successfully applied to severe asthma and has allowed the identification of phenotypes with clinically important differences (9). Perhaps a similar approach is required for the optimum assessment and management of athletes with exercise-related symptoms.

1.2.3 Diagnosis of asthma in elite athletes

The Medical Committee of the International Olympic Committee introduced restrictions in the use of asthma medications at international competitive level as long ago as 1993. Since then these rules have been reviewed on several occasions and there is now a consensus of opinion between the IOC-MC (responsible for Olympic sports) and the World Anti-Doping Association (all international sports) concerning the restriction of the use of these medications in competition. Whereas previously a simple athlete declaration was sufficient, concerns about the increased use of medications in sport (especially the Olympic Games) and evidence that symptoms are not good predictors of disease (24) has led to criteria requiring objective evidence of airways dysfunction using reversibility or provocation testing. Acceptable tests, their diagnostic criteria and their advantages and disadvantages are summarised in table 1.2 (21).

Table 1.2: A summary of the various tests approved for the diagnosis of exercise induced bronchoconstriction by international drug testing organisations.

Test	Positive test criteria	Advantages	Disadvantages
Reversibility to β-agonists	Reversibility in FEV ₁ of >12% in response to permitted β -agonist	Shows immediate response to therapy Easy to perform	Low sensitivity in athletes
Exercise	A drop of >10% in FEV ₁ on exercise challenge	Real stimulus High positive predictive value Common trigger for an asthma attack Many mediators involved	Ergometers are expensive and space occupying Inspired air needs to be 'dry' Requires 6–8 min of vigorous exercise Sensitivity can be low Single 'optimal' stimulus so severe responses (>50%) can occur
Methacholine	Dose creating a drop in FEV ₁ of 20% (PC20 <4mg/ml or PD20 < 2 umol)	Sensitive test Dose–response curve obtained Negative test in symptomatic patients is useful to exclude asthma FDA approved formulation available	Response is only being tested to one mediator Different cut-off points used to identify hyperresponsiveness Positive test not specific for asthma Positive test occurs with airway injury Solutions need to be prepared and nebulizers calibrated
Mannitol	Dose (<635mg) causing a drop in FEV ₁ of \geq 15% (PD15)	Available as a convenient and standardized test kit Dose–response curve obtained Positive test predicts active asthma and potential for EIA More than one mediator involved	May not be as sensitive as direct challenges to identify BHR in some population groups Some cough during challenge
Eucapnic voluntary hyperventilation	A drop of >10% in FEV ₁ post challenge	High sensitivity to identify EIA Negative test highly likely to exclude EIA Equipment less expensive compared with exercise	Special gas mixture needed (5% CO ₂ , 21% O ₂ , 74% N ₂) Expensive to have a commercially prepared gas mixture Less sensitive when test duration is less than 6 min yet a 6 min protocol can provoke a severe response (>50%)

These different challenge tests have different measurement characteristics which we need to consider when interpreting the results. In particular it is useful to draw a distinction between tests such as inhaled histamine and methacholine, which act directly on airway smooth muscle and assess airways hyperreactivity only, and indirect challenge tests such as exercise, eucapnic voluntary hyperpnoea and mannitol, which involve an intermediary pathway such as mast cells or neuronal pathways and thus may provide a more complete assessment of the asthmatic process.

Methacholine and histamine are routinely available in hospital practice but due to safety concerns are not freely available outside of the hospital environment. Exercise testing facilities are expensive and require specialised equipment and expertise, making sport specific testing more attractive. Similarly the eucapnic voluntary hyperventilation testing equipment is cumbersome and expensive. The mannitol challenge test kits come as a portable inhaler pack and have proven safety data making them a good prospect for testing in the pre-hospital environment.

Photo 1.1: Mannitol testing kit.



Photo 1.2: Eucapnic voluntary hyperventilation testing kit from Anderson et al 2001(59)

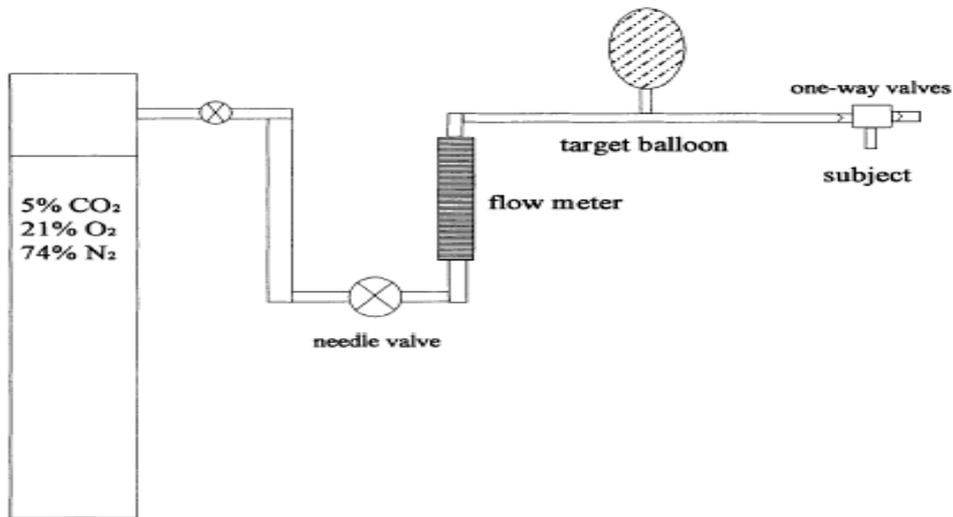


FIGURE 1. Schematic of EVH apparatus. Dry gas is metered through a rotameter, then fills the target balloon. The subject is coached to maintain a \dot{V}_E sufficient to keep the balloon half emptied.

Methacholine bronchoprovocation test:

The methacholine bronchoprovocation test is usually carried out in a hospital clinical setting. Increasing doubling concentrations of drug are delivered either by the tidal breathing or dosimeter method and the concentration or dose that creates a 20% fall in FEV₁ from baseline is expressed as the PC or PD20.

The methacholine challenge test has been well validated as a direct challenge to the airways smooth muscle (60) and is the most widely used bronchoprovocation test, recommended in the latest version of the British Thoracic Society guidelines as the challenge test of choice in those patients with an intermediate probability of asthma and normal or near normal spirometry (7). Of all the tests available in this setting it has the best validity, with a sensitivity of greater than 90%. This means that a negative test provides strong evidence against asthma in a symptomatic patient (61). Concerns remain about using the dosimeter

technique and the effect of deep inspiration induced bronchoprotection but these are more theoretical concerns than real issues. This test has fallen out of favour as a screening test in athletes due to the large number of false positives (23), but it remains a very useful clinical test because of its high negative predictive value.

Exercise and sports specific exercise testing:

Laboratory exercise testing has been well established as an indirect challenge test and has the benefits (and drawbacks) of assessing the individual during the activity in which they complain of symptoms. There needs to be environmental control of both air temperature and relative humidity to standardise results and monitoring of continuous heart rate to determine an appropriate challenge. The test should challenge the athlete to greater than 85% maximum heart rate for a period no longer than 6-8 minutes as longer testing may miss mild bronchoconstriction. A warm up should be avoided to prevent the induction of refractoriness. Traditionally these use either a treadmill or cycle ergometer and assess whole body exercise. However, in the elite athlete population it is felt that standard exercise challenge has a low sensitivity for detecting airways dysfunction (62) because athletes train at such high and prolonged intensity that standardised laboratory exercise protocols fail to sufficiently tax them to the level required to induce bronchoconstriction. In addition the laboratory environment is not as austere as the environment in which they train and compete (63). Sport specific exercise tests take the athlete in to the natural sporting environment and protocols are developed on a sport by sport basis, reflecting the complexities and challenges of each sporting discipline. These specific exercise challenges more accurately reflect the training environment but again often fail to induce bronchoconstriction due to factors that are difficult to standardise, such as humidity and temperature (64). These problems have led to the development and use of substitute tests to aid with the diagnosis.

Eucapnic Voluntary Hyperventilation (EVH)

The EVH test was developed to imitate the effects of ventilation at high flow rates on the airway surface liquid, creating a drying and osmotic effect (59). It relies on the athlete being able to attain and maintain a ventilatory flow equivalent to 85% of maximum voluntary ventilation (approximately $30 \times FEV_1$) for 6-8 minutes breathing a gas mixture containing 5% carbon dioxide to maintain eucapnia. It is a laboratory based test that creates an 'all or nothing' response making it useful in those with normal or supranormal spirometry but questionable in those with abnormal resting spirometry. The diagnostic criteria used by the IOC-MC (ie a 10% drop in FEV_1 post challenge) make it a very sensitive test for the diagnosis of airways dysfunction in athletes (62). This has led to the adoption of the EVH test as the 'gold standard' test by the IOC-MC and WADA and it has been used as a screening test at elite level in some countries (65). One concern is that the criteria for a positive test have been set too low, meaning that relatively few subjects identified have genuine steroid responsive airway pathology (66).

Mannitol

Mannitol is a dry powder inhalation test that acts by developing an osmotic gradient across the airway epithelium, thereby triggering bronchoconstriction by a mechanism similar to that responsible for EIB (62). However, in a recent large multi-centre study it was found to be no more sensitive or specific than a methacholine inhalation test for the diagnosis of EIB determined on laboratory exercise testing (67). It comes as a standardized test kit, which contains pre-filled mannitol capsules in escalating doses and a hand-held dry powder inhaler device. It can be used safely in most clinical settings and may be useful in the pre-hospital environment. It has been widely used in the diagnosis of EIB and has helped to determine

some of the underlying mechanisms involved, most notably the involvement of mast cells (34;68). Mannitol is yet to be approved in the US for this form of testing.

1.2.4 Clinical aspects of asthma in elite athletes

Alternative diagnoses in athletes

There are several important differential diagnoses that need to be considered in athletes presenting with respiratory symptoms. These conditions are important considerations for anyone involved in assessing and managing the respiratory health of athletes.

A frequently seen alternative diagnosis is vocal cord dysfunction, where the symptoms are often clearly related to exercise and may be indistinguishable from exercise asthma.

However, the presence of inspiratory stridor (occurring during maximal exercise and stopping when the exercise is terminated) helps to determine the diagnosis. This is often mistaken for wheeze and definitive challenge testing may be required to establish the diagnosis. It is most often seen in young female athletes (69) and has an incidence in elite athletes of around 5% (70). Half of patients with vocal cord dysfunction also have EIB (70) which complicates assessment. The diagnosis can be confirmed by detecting impaired inspiratory flow on a flow-volume loops either spontaneously or after an airway provocation test, but these can be insensitive. Direct laryngoscopy with an appropriate stressor may also be diagnostic.

Management of the condition usually involves the use of behavioural therapy by a speech and language specialist using a combination of breathing techniques to recognise an impending event and avert or control this (71).

Another common alternative diagnosis is dysfunctional breathing. This is often associated with mild underlying asthma and can be difficult to distinguish from true asthma symptoms

unless specifically addressed. Patients tend to have symptoms, elevated anxiety, poor asthma control scores and breathlessness during usual daily activities in excess of that expected by the identified airways dysfunction. It is common throughout the asthma population and although it is felt to be as common in the elite athlete population most literature refers to individual cases and there are no robust epidemiological data to help support this. It can be treated with simple physiotherapy delivered breathing control techniques (72) and like vocal cord dysfunction is refractory to traditional asthma medication.

Rarer causes of symptoms in elite level athletes include swimming induced pulmonary oedema, which presents as severe dyspnoea or cough immediately following swimming (73) and exercise induced hypoxaemia, which occurs in elite endurance athletes who display significantly compromised pulmonary gas exchange on heavy exercise (74).

Treatment of asthma in elite athletes

Bronchodilators

Quick-acting (beta)₂-selective, adrenergic agonists administered by inhalation are the most effective therapy for rapid reversal of airflow obstruction and prompt relief of asthmatic symptoms. When used before exercise, β -agonists offer protection against subsequent bronchoconstriction and are therefore, the most studied and used medication in this respect (75). The short acting inhaled β -agonists have an almost immediate effect and last for a maximum of 4 hours (76). Newer long acting β -agonists have a longer duration of action but concerns about the development of tolerance to these medications (77) and potential safety issues (78) means they are not recommended for regular use in the treatment of exercise induced bronchoconstriction, even if used with concurrent corticosteroids (79). However, more recent work on the mechanisms of these adverse effects have shown a clear reversal by

concomitant steroid usage (80) and fears for the safety of these drugs when used in conjunction with inhaled corticosteroids have not been borne out by large population based studies (81).

Inhaled corticosteroids

Corticosteroids have proved effective in the treatment of asthma, as they have in many other inflammatory diseases, because of their multiplicity of anti-inflammatory activities. Along with suppression of airway inflammation, non-specific airway hyperresponsiveness typically decreases by a factor of two to four resulting in improvements in asthma symptoms.

There have been few studies of the effects of inhaled corticosteroids on exercise induced bronchoconstriction in athletes and no studies in top-level athletes. One study looked at young competitive skiers with airways symptoms and airways dysfunction but failed to show any response to inhaled budesonide over a 10-32 week period, although the numbers in each group were small and the study underpowered (39).

Much of the evidence for the use of these medications has been extrapolated from studies done in children, where there has been a marked response in symptoms to inhaled steroids at an early stage, but direct challenge by methacholine remains positive for many months (82). This probably reflects the response of underlying eosinophilic inflammation (38) and may not be representative of the situation in adult athletes.

Leukotriene antagonists:

The cysteinyl leukotriene-receptor antagonists block the action of leukotriene C4, D4, and E4 at the type 1 cysteinyl leukotriene receptor inducing bronchodilation but having a variable effect on airway inflammation. There have been only a few studies looking at the modulating effect of montelukast on EIB in adult athletes. In one study no effect was found on airways dysfunction, exhaled nitric oxide, and sputum cell parameters in 16 ice-hockey players (83), whilst another reported protection from eucapnic voluntary hyperventilation induced bronchoconstriction in a randomised double-blind placebo-controlled cross-over study in 11 subjects (84). It may have an effect in some athletes, but not all.

Disodium cromoglycate and nedocromil sodium:

The cromoglycates work by stabilising mast cells and reducing mast cell secretion of inflammatory mediators. These drugs have been shown to offer some protection when taken immediately before exercise (85) and this may be through the stabilisation effect on mast cells which are thought to be activated by the osmotic changes in the airway surface layer (32). There have been no randomised controlled trials in athletes and with the increasing use of inhaled corticosteroids these medications are used less frequently in clinical practice.

1.2.5 Airways inflammation in elite athletes

Over recent years there have been significant improvements in the phenotyping of asthma in the clinical population. This approach has aimed to determine the differing levels of airways dysfunction and airways inflammation that are present in an individual presenting with airways disease and to tailor appropriate therapies to treat this effectively. Central to this approach has been the use of ‘inflammometry’ in the assessment of airways inflammation to phenotype disease in to eosinophilic or non-eosinophilic disease. This is important because we know that the presence of eosinophilic airway inflammation is more closely predictive of

a response to corticosteroid therapy than any other marker of disease (86;87) and that there is only a weak correlation between the presence of eosinophilic airway inflammation and either the pattern or severity of airway dysfunction (88). We also know that exhaled nitric oxide is a good predictor of underlying eosinophilic airways disease (89) and this gives us a very practical non-invasive means to assess airway inflammation. The potential to use these techniques for the phenotyping of asthma in elite athletes has not been investigated to any degree.

In the normal asthma population symptoms on exercise are seen as a reflection of poor asthma control and an increase in asthma therapy improves control, lessens symptoms and improves exercise capacity (7;90). This is the basis for the control centered guidelines in use throughout much of the developed world (7;91) and relies on the supposition that asthma symptoms have a concordant relationship with treatable aspects of airways disease such as airways inflammation. Recently this phenomenon has been clearly demonstrated in a study of asthma patients with eosinophilic airways inflammation (38). The response to exercise challenge testing was directly proportional to the degree of eosinophilic airways inflammation and the response to increased therapy (inhaled corticosteroids) was also proportional, with those patients with the highest levels of airways inflammation demonstrating the most significant responses to therapy and the best improvements following repeat exercise challenge. This has been supported recently by a detailed study looking at the response to inhaled corticosteroids in a large asthma cohort and relating this to the presence or absence of eosinophilic airways inflammation (92). Once again the response to inhaled corticosteroids was clearly related to the presence of markers of eosinophilic airways inflammation.

However, within the severe asthma population, detailed disease phenotyping has shown a significant decoupling of symptoms from either markers of airways inflammation or measurements of airways dysfunction (9). Here, more detailed patient assessments have allowed the characterization of patient's airways disease and have made it possible to tailor therapy to the predominant disease phenotype, notably inflammation predominant, concordant or symptom predominant disease (93). This has clearly demonstrated a high degree of heterogeneity within this more complex disease group and suggests that for some patients, simple guideline directed therapy may not be appropriate.

We already know that within the elite asthma population there is a significant degree of heterogeneity in terms of airways inflammation (40) and a similar disconnect between symptoms, measurements of airways dysfunction and airways inflammation (94). Symptom questionnaire studies have shown a high level of symptoms in athletes with no demonstrable pathology on subsequent challenge testing and the absence of symptoms in others with high levels of airways dysfunction (24). Airways inflammation seems to vary enormously between individuals and in those from different sporting backgrounds (94;95) with little or no steroid responsiveness seen in some specialized groups (39). More recently, an invasive study of airways inflammation has shown patterns of disease in athletes akin to those seen in normal asthmatic patients, but with little correlation between airways inflammation and demonstrated airways hyperresponsiveness (94).

It has also been proposed that athletes have a unique form of airways inflammation / injury that is a direct consequence of their sports participation, with high airflow across the lungs creating epithelial shear stresses and damage that leads to airways hyperreactivity (58). In these high performance, endurance individuals there is little evidence of eosinophilia or

steroid responsiveness but marked evidence of airways hyperreactivity (96). This process is felt to be primarily related to mast cell activation (97) and suggests that in some individuals (and for some sports) sporting activity at high level may even be injurious or detrimental to the airways.

The application of 'inflammometry' techniques in the elite athlete population would allow for more definitive delineation of any underlying disease phenotypes within this group and in particular to assess the degree of eosinophilic airways inflammation, its relationship to airways hyperreactivity and how best to test for these factors in symptomatic athletes. In particular, heterogeneous responses to direct and indirect airways challenge tests have not been fully investigated (98) nor have levels of variability in the degree of eosinophilic airways inflammation (40). Development of substitute tests for exercise as the means to provoke bronchoconstriction were carried out many years ago, prior to advances in 'inflammometry' and so failed to delineate the differences seen between eosinophilic or non-eosinophilic individuals. In particular the development of EVH (59) and mannitol (99) as clinical tests, involved contrasting response to these new tests against patients who had significant drops in FEV₁ on exercise testing, i.e. individuals we now know have a very high chance of being markedly eosinophilic (38). This is reflected in further demonstration of steroid responsiveness in these patients in later studies (68) and correlating this to test responsiveness in much the same way as has recently been demonstrated with exercise. This suggests that these tests should be good at delineating eosinophilic airways inflammation, but this may not be entirely relevant to athletes, many of whom have non-eosinophilic airways inflammation and no symptoms except on extremes of exertion. How these patterns of inflammation relate to steroid responsiveness also requires further attention.

1.2.6 The mast cell and exercise induced bronchoconstriction

There is increasing evidence that epithelial stress and damage play a significant role in the development of airways hyperreactivity in athletes (100). How this epithelial damage leads to airways hyperreactivity remains the subject of much conjecture. Certainly it has been recently demonstrated that repetitive bronchoconstriction can influence epithelial structure (101) and that patterns of airways remodeling is seen in elite level athletes even without demonstration of increased airways responsiveness. It has been speculated that with high levels of non-eosinophilic airways inflammation in this group that the mast cell is the key effector cell in exercise induced bronchoconstriction in elite athletes. This would suggest an interaction between epithelial cell stress / damage and mast cell activation that exists irrespective of the acquired immune response or presence of atopic sensitization.

The human lung mast cell and its role in asthma pathogenesis has been described in great detail over recent years (102-104), but despite these advances, many questions related to their function remain unanswered. Mast cell mediator release has been clearly demonstrated in bronchoconstriction related to allergic asthma (105), aspirin sensitization reactions (106) and exercise asthma (107), and more recently has been demonstrated in relation to airways responses to indirect challenge testing (108;109). However, how airways challenge / stress leads to mast cell mediator release and whether this is an effector or bystander to bronchoconstriction, remains unclear.

We know from studies of comparative patterns of airways inflammation that mast cell position in the airway tissues is very important (37) and that their interaction with the airways epithelium may have a significant role to play in mast cell regulation (57), something that may then be lost when the epithelial layer is either stressed or damaged. However, how this

relates to airways eosinophilia or perhaps more importantly, lack of eosinophilic airways inflammation, remains unclear. In particular, how recurrent airways epithelial stress leads to the development of airways inflammation, airways remodeling and airways hyperreactivity needs further investigation. Of equal import is the degree to which this process can be counteracted either therapeutically or naturally. In particular, whether the development of airways hyperresponsiveness is related to the gain of a negative action within the airways or the loss of a protective (bronchoprotective) mechanism and whether this protective mechanism can be enhanced to prevent the negative outcomes. Of note, previous work with prostaglandin E2 suggested a strong bronchoprotective role in the human airway (110;111) and initial clinical trials with this medication were promising, however, it's role was not supported by more recent in vitro work (57).

Therefore, the role of the mast cell in demonstrable airways hyperreactivity; its relationship with epithelial function or injury; its role in the airways response to direct and indirect challenge testing and the best therapeutic mechanism for ameliorating these functions all requires more detailed investigation both in vivo and in vitro.

1.2.7 Remaining questions

The extent of eosinophilic airways inflammation seen in elite athletes, it's relationship to measured airways hyperreactivity and the degree of epithelial cell damage, mast cell activation and the response of all these elements of disease to traditional anti-inflammatory treatments (such as inhaled corticosteroids) needs to be fully elucidated. Characterising phenotypes of disease within the elite athlete population would enable tailored therapeutic intervention in this group of high achieving individuals and might allow some insight in to

mechanisms of airways inflammation and injury, and possible preventative or therapeutic strategies that can be implemented.

It should also be possible to further investigate the refractory period; in particular whether it occurs as readily in elite athletes as it does in asthmatics who bronchoconstrict with exercise challenge, and what determinants make it more likely to occur. Investigating the mediator profile in the airways after exercise challenge may allow us to further investigate the pathological differences between elite athletes, asthmatics and non-athletes, and by relating this to refractoriness, elicit those factors that are necessary for bronchoconstriction or its prevention.

Table 1.1: Prevalence of exercise related bronchoconstriction in elite level athletes.

Adapted from Carlsen et al (21).

Authors	Diagnostic method	Sport	Number (n)	Prevalence(%)
Voy RO	Questionnaire	Olympic Summer athletes	597	11%
Weiler JM et al	Questionnaire	Olympic Winter athletes	196	22.5%
Helbing et al	Questionnaire	Swiss athletes	2060	7.1%
Larsson K et al	BHR (methacholine) and symptoms	Skiers	42	55%
Heir T and Oseid S	Questionnaire	Skiers	153	14%
Weiler JM et al	Questionnaire	Olympic summer athletes	699	>20%
Sue-Chu M et al	BHR (methacholine) and symptoms	Skiers	171	BHR: 14-43% Symptoms: 43-55%
Provost-Craig et al	Sport specific exercise test	Figure skaters	100	30%
Mannix ET et al	Sport specific exercise test	Figure skaters	124	35%
Mannix ET et al	Sport specific exercise test and eucapnic hyperventilation	Figure skaters	29	55%
Feinstein RA et al	Exercise test	Male footballers	48	19%
Sodal A	BHR (methacholine)	Female soccer	17	35.5%
Ross RG	Reversibility	Canadian footballers	34	56%
Schoene RB et al	Exercise test	Track and field	Male 50 Female 23	10% 26%
Helenius IJ et al	Questionnaire	Track and field	Endurance 107 Power 106	17% 8%
Helenius IJ	Questionnaire, BHR (histamine)	Swimmers	29	48%
Langdeau et al	BHR (methacholine)	Various sports		49%
Rundell KW et al	Questionnaire Sports specific exercise test	Cold weather athletes	158	26%
Wilber RL et al	Sport specific exercise test	Cold weather athletes Cross country skiers	170 58	23% 50%
Maiolo et al	Questionnaire	Italian summer athletes	1060	15%

2.0 Hypothesis and Aims

2.1 Hypothesis

I hypothesise that, within the population of athletes that describe respiratory symptoms on exercise or have positive results to the variety of airways challenge tests currently used, there is significant heterogeneity with respect to levels of airways inflammation and airways hyperreactivity. There may be sport specific phenotypes of disease within this specialized patient group, and that by identifying these and by defining these phenotypes accurately in terms of their pathophysiology, we will be able to offer improved diagnostic testing for elite athletes and streamline and improve the treatment of their performance limiting symptoms.

I also hypothesise, that damage to the airway epithelium is a key factor in the development of airways hyperresponsiveness in elite athletes. I will model this in an in vitro system where human airways epithelium inhibits mast cell mediator release both constitutively and in response to IgE mediated secretion. I will attempt to determine whether this:

- a) Is due to a soluble mediator
- b) Is independent of cell-cell interaction
- c) Is due to a low molecular weight lipid mediator
- d) Acts through a G-protein coupled receptor system

2.2 Aims:

1. Clinical and pathophysiological assessment of exercise induced bronchoconstriction:
 - a. To compare and contrast the different direct and indirect airways challenge tests currently used in the diagnosis of exercise induced bronchoconstriction
 - b. To determine how the responses to these various challenges tests relate to patterns of underlying cellular airways inflammation, exhaled nitric oxide and mediator release
 - c. To compare and contrast airways inflammation and hyperresponsiveness in pool and non-pool based elite performance athletes

2. Exploring the interaction of airways epithelial cells and human lung mast cells and their potential relevance to exercise induced bronchoconstriction:
 - a. To examine airways epithelial-mast cell interactions
 - b. To explore these interactions for evidence of soluble mediators that act as bronchoprotective factors and modulate mast cell function
 - c. To explore the role of these bronchoprotective factors in both healthy and asthmatic or injured epithelium and to determine how these might be modulated to therapeutic benefit

3.0 Methods

3.1 Clinical Studies

3.1.1 Subjects

One hundred and forty endurance athletes, all exercising for more than 15 hours per week, from a variety of sporting backgrounds agreed to take part in the trial. All were competing at international level and had expressed symptoms suggestive of exercise asthma and had been previously using medication whilst competing under a therapeutic use exemption (under the then current guidance). They were all either inhaled corticosteroid naïve or had withdrawn inhaled corticosteroids and long acting beta-agonists for at least 4 weeks prior to starting the study. All challenge tests were carried out at least 48 hours apart and following an 8 hour interval from previous exercise, short acting beta-agonist use or caffeine. Pool based athletes trained for at least 5 hours per week in an indoor pool environment and non-pool based athletes trained in an indoor pool based environment for <0.5hrs per week.

3.1.2 Study design

The study was performed at the Respiratory Research Unit, Institute for Lung Health, Glenfield Hospital, Leicester and at the Department for Human Sciences, University of Loughborough, Loughborough, UK. Participants visited the research units on four separate occasions. An initial assessment visit included a physical examination, detailed medical history questionnaire, Juniper asthma control questionnaire (ACQ), Asthma Quality of Life Questionnaire (AQLQ), exhaled nitric oxide (FE_{NO}) measured using the Niox MINO, baseline spirometry, a methacholine PC₂₀, skin prick tests against common aeroallergens (dog, cat, HDM, grasses, tree, aspergillus, histamine control) and an induced sputum differential inflammatory cell count was obtained at each visit. Visit 2 entailed measurement of FE_{NO}, spirometry and a eucapnic voluntary hyperpnoea (EVH) provocation test. Visit 3

measured FE_{NO}, spirometry and a mannitol provocation test. Visit 4 measured FE_{NO}, spirometry and an exercise challenge test. In order to avoid any effect of bronchial provocation with one agent on the outcome of provocation with another agent, there was an interval of at least 48 h between challenges. The study was approved by the local regional ethics committee (Study UHL 10589); all gave written informed consent.

3.1.3 Measurements

Single flow FE_{NO} was recorded at 50 ml/s as previously described, using the Niox MINO (Aerocrine, Solna, Sweden); the mean of two acceptable readings was taken, with at least 30s of relaxed tidal breathing between manoeuvres (112). Spirometric parameters were measured using an electronic spirometer (Micro Loop, Micro Medical, Cardinal Health, UK) according to guideline recommendations. Asthma quality of life was measured using the Juniper asthma quality of life score (AQLQ) (113) and asthma control using the Juniper asthma control questionnaire (ACQ) (114), a further sports related questionnaire modified from one used in previous research studies in elite athletes was also used (115). This focused on respiratory symptoms at rest and on exercise, use of asthma medication, history of doctor diagnosed hayfever, allergy and asthma and childhood disease history. This was used to explore the symptoms which best predicted disease in the elite athlete population and we created a total symptom score at rest and on exercise. Sputum induction and processing was carried out using our standard methods (10;87). The cut-off point used for the level of sputum eosinophils was 3% based on the level of eosinophils that has been shown to best predict a response to treatment with steroids (116). Cell counting was performed by an experienced observer blind to the clinical characteristics of the subjects.

3.1.4 Challenge tests:

Methacholine PC20: The methacholine PC20 was measured using the standard tidal breathing method with a maximum inhaled concentration of 16 mg/ml, as previously described (117). Methacholine PC20 was calculated by linear interpolation of the change in FEV₁/concentration of methacholine on a log dose-response curve as the inhaled concentration of methacholine causing a 20% reduction in FEV₁. Non responders were given a nominative PC20 value of 16mg/ml, though results were analysed from the dose response curve as well.

Mannitol: Bronchial provocation testing using mannitol powder (Aridol™; Pharmaxis, UK) contained in capsules and inhaled from an Osmohaler™ dry powder inhaler (Plastiap, Osnago, Italy) was performed up to a cumulative dose of 635 mg. Based on the findings in healthy non-asthmatics, a 15% decrease in FEV₁ to 635 mg or less is regarded as a positive response (118;119) and indicates airways hyper responsiveness. The cumulative dose of methacholine or mannitol required to provoke a 15% fall in FEV₁ (PD₁₅), was calculated by interpolation of the log-linear dose–response curve. Non-responders were given a nominative PD₁₅ of 650mg.

Eucapnic voluntary hyperventilation: Bronchial provocation challenge testing with eucapnic voluntary hyperventilation (EVH) was then performed as per previously described standards (120). Participants breathed a mixture of dry compressed gas (5.0% CO₂, 21.0% O₂, balance N₂) at a target rate of 85% of their maximum voluntary ventilation (MVV) per minute (calculated as 30 times the baseline FEV₁, which approximates 85% MVV) for 6 min. Gas was channeled from a cylinder into a calibrated multistage flow compressor converter (GCE EN ISO 7291 / EN ISO 2503) and then through an inspiratory target balloon (medical research bladder 100 L) that was maintained half full (to ensure correct minute ventilation)

and then to a two-way, low resistance valve (Harvard Apparatus, 2-Way Non- Rebreathing Valve) and mouthpiece (Harvard Apparatus) and exhaled gas measured using a standard dry gas meter (Harvard Apparatus, Kent, UK). A metronome, timed for 30 cycles per minute, was used to help guide the athlete to achieve their target MVV. Spirometry was performed at 1, 3, 5, 7, 10, 15, 20, and 30 min after the EVH challenge.

Urine: The athletes were encouraged to drink freely before the test and afterwards during recovery. At 60 minutes post the start of the EVH test a urine specimen was attained for mediator metabolite assay.

3.1.5 Assays

Sputum supernatants were prepared as described and sputum mediator concentrations were measured using commercially available enzyme linked immune-sorbent assays according to the manufacturers agreed protocols. Histamine (Cayman Chemicals, Beckman Coulter UK Ltd, cat IM2562), PGE2 (Cayman Chemicals, R&D Systems Europe Ltd, KGE004B), PGD2 (Cayman Chemicals, Cayman Europe, 512021) and Cyst-Lt (Cambridge BioScience, 900-070) were measured in the sputum supernatants from the post test induced sputum and 9α - 11β -PGF2 (Cayman Chemicals, Cayman Europe, 11β PgF2 α kit, 516521.1) was measured in a urinary specimen collected 60 minutes post test. Subjects voided their bladders immediately before testing, had free access to water throughout the tests and only voided their bladders at the 60 minutes post test collection. Urinary creatinine concentration was measured using our hospital laboratory standard technique and expressed as mmol/L (JAFFE kinetic blank ratio concentration). The sensitivity levels of the assays were as follows: histamine 50×10^{-3} ng/ml; PGD2 3.2×10^{-3} ng/ml; PGE2 8.25×10^{-3} ng/ml; cyteinyl-leukotrienes 13×10^{-3} ng/ml.

The intra-assay coefficient of variability of the assays was 5-10% and the inter-assay coefficient of variability was 3-15% across the range of mediators measured.

3.1.6 Statistical methods

Graph Pad Prism (version 5) and SPSS version 14 were used for data analysis. The FE_{NO} and sputum eosinophil counts values were log normalized as has been previously described (87). Methacholine PC₂₀ and mannitol PD₁₅ values were analysed with a nominative negative value of 16mg/ml and 650mg respectively and as the dose extrapolated from the dose/response slope. Mediator levels were converted to ng/ml and then analysed both as absolute values and the log normalized values. Mediator levels were converted to ng/ml and then analysed both as absolute values and the log normalized values. Urinary metabolite levels have been corrected for creatinine concentration as has been previously described (121).

3.2 Laboratory studies

3.2.1 BEAS-2B cell culture

The BEAS-2B epithelial cell line was purchased from the European Collection of Animal Cell Cultures (Porton Down, Wiltshire, UK). Cells (passages 8–12) were grown on human plasma fibronectin-coated T75 culture flasks in BEBM media (Clonetics Cat. No. CC4175), with an added enhancement bullet kit (Clonetics Cat. No. CC4175), Pen/Strep (5ml) and fungizone (5ml) to create basal epithelial growth media (BEGM). BEAS-2B were then passaged on to human plasma fibronectin-coated 16-well 0.40 µm Transwell plates and then grown to confluence prior to use in assays.

3.2.2 HLMC purification and culture

All subjects donating lung tissue gave written informed consent, and the study was approved by the Leicestershire Research Ethics Committee. HLMC were dispersed from macroscopically normal lung obtained within 1 h of resection for lung cancer using immunoaffinity magnetic selection as described previously (122). Final mast cell purity was >99%, and viability >99%. HLMC were cultured in DMEM, 10% FCS, antibiotic / antimycotic solution, SCF 100 ng/ml, IL-6 50 ng/ml and IL-10 10 ng/ml (122).

3.2.3 Air-Liquid interface cultures

Asthmatic subjects (n=6) and healthy controls (n=6) were recruited from Glenfield Hospital, Leicester, UK. Asthmatic subjects had a consistent history and objective evidence of asthma, as described previously (37). Subjects underwent extensive clinical characterization including video-assisted fiberoptic bronchoscopic examination. The study was approved by the Leicestershire Ethics Committees. All patients gave their written informed consent. Primary epithelial cells were isolated from bronchial brushes, grown to confluence on 1% PureCol-coated surfaces (Inamed Biomaterials, Nutacon, The Netherlands) as submerged cultures using bronchial epithelial growth medium (BEGM, Lonza Verviers, Belgium) supplemented with 0.3% Fungizone® antimycotic (Gibco, Invitrogen, Paisley, UK) and 1% antibiotic-Antimycotic (AA) (Gibco). When confluent, epithelial basal cells were seeded into PureCol-coated Transwell® inserts (12mm diameter, 14µm polyester membrane) (Corning, Lowell, MA), firstly in submerged culture to confluence, secondly in Air-Liquid-Interface (ALI) using ALI medium (50:50 BEGM:DMEM [Gibco], supplemented with 0.3% Fungizone®, 1% AA and 100nM retinoic acid [Sigma, Poole, UK]). Ciliated cultures with high ciliogenesis were used for experiments.

3.2.4 Co-culture experiments:

HLMC-BEAS-2B co-culture:

Three conditions were compared initially: i) HLMC (5×10^4) in direct contact with confluent BEAS-2B cells cultured in 24 well plates, ii) BEAS-2B grown to confluence initially on an inverted $0.4 \mu\text{m}$ Transwell membrane which was then inserted into the plate in the correct orientation, with HLMC (5×10^4) then added to the top chamber, and iii) HLMC (5×10^4) cultured alone on plastic in the bottom of a 24-well plate. The cells were cultured for 16 h, the media then removed gently, any non adherent cells in the removed media recovered by centrifugation and the cells returned to their respective wells in fresh media. They were then activated with anti-human IgE for 30 minutes, supernatants removed and the cells recovered by centrifugation. The remaining cells were then lysed with sterile water to allow for measurement of cell histamine content.

In further experiments, BEAS-2B were grown to confluence in the top of a Transwell membrane, and HLMC added to the bottom chamber.

HLMC-ALI AEC co-culture:

These were carried out exactly as the latter BEAS-2B experiments with ALI AEC cultures grown to confluence as described above and then transferred into a fresh well with HLMC added to the bottom chamber.

Mast cell activation

Mast cells were sensitised using 2.5 ng/ml human IgE (Calbiochem, Darmstadt, Germany) for at least 1 h, washed and re-suspended in fresh media. They were stimulated with anti-IgE (Mouse IgG Hybridoma Reagents, Baltimore, USA) at a final concentration of 1/1000 for 30 minutes. Pertussis toxin (PTX), (Sigma, UK) was used at a final concentration of 500 ng/ml,

the EP₂ receptor antagonist AH6809 (Sigma, UK) was used at a final concentration of 10 μM based on our previous experience with this (123). AEC wounding was carried out by placing a simple X on the cultured cells with a 10 μl pipette tip.

3.2.5 Mediator assays

Histamine was measured by sensitive radioenzymatic assay as described previously (122).

Lipoxin A₄ was measured by ELISA (Oxford Biomedical Research, Oxford, MI, USA) according to the manufacturer's instructions.

3.2.6 Fractionation of AEC supernatants and co-culture supernatants

We fractionated AEC supernatants from primary AEC monolayers using reversed phase C¹⁸ solid phase extraction (SPE)(Phenomenex Strata-X, 33 μm, 85 Å, <10 kDa) (Phenomenex, Torrance, CA, USA) to remove small lipid mediators (124). The cartridge was conditioned with methanol followed by water prior to the application of the supernatant. The lipid-depleted AEC media was collected then incubated with HLMC and the inhibitory effect on constitutive histamine release was investigated.

3.2.7 Measurement of resolving mediators using liquid chromatography (LC)-mass spectrometry

We used liquid chromatography (LC)-mass spectrometry (MS) to look for the presence of resolving mediators of inflammation (lipoxins, resolvins, protectins, maresins) in both primary AEC monoculture supernatants and HLMC-co-culture supernatants. For targeted determination, cell culture media were subject to C¹⁸ solid phase extraction (see above), which purifies lipid mediators and removes interfering substances. The extracted samples were kept as solutions in methanol at -20 °C; these were then dried by rotary evaporation and

reconstituted in LC solvent prior to analysis. Samples were analysed using a Waters (Manchester, UK) Acquity ultra performance (UP)LC system coupled to a Waters Xevo TQ triple quadrupole mass spectrometer operated in negative ion mode with selected reaction monitoring. *Chromatography*: Solvents were 0.02% acetic acid (solvent A) and 45% acetonitrile (solvent B), used in a gradient system varying between 50:50 A:B and 20:80 A:B. The column was a Waters Acquity BEH C₁₈ 1.7 µm, 2.1x150 mm. Injection volume was 10 µl, total run time 17 minutes. *Mass spectrometry*: Capillary voltage was 2.6 kV, cone voltage 25 V; desolvation temperature was 550 °C with gas flow 800 l/hr. Collision gas flow was 0.2 l/min. Ion transitions monitored for selected reaction monitoring were: resolvin D1 and D2 375.2 → 141.2; lipoxin A₄ 351.2 → 115.2; deuterated-lipoxin A₄ 356.2 → 115.2; resolvin E1 349 → 195; resolvin E2 333.2 → 199.2; protectin D1 359.2 → 206.2; maresin 1 359.2 → 250.2 (125;126). Deuterated internal standard (d5-lipoxin A₄) was added to the media, enabling the accurate quantitative determination of each mediator.

4.0 Studies

4.1 Airway inflammation and the response to direct and indirect airways challenge testing in elite endurance athletes with symptoms suggesting exercise-induced asthma

Abstract:

There is a high prevalence of exercise induced bronchoconstriction in elite performance athletes. Sporting regulatory bodies advocate use of objective tests for diagnosis, but how response to these relates to airways inflammation is unclear. We investigated responses to standard direct and indirect challenge tests and patterns of airways inflammation in symptomatic, international level, endurance athletes.

Seventeen endurance athletes who were all either inhaled corticosteroid naïve (or had withdrawn inhaled corticosteroids for 4 weeks) were recruited. They underwent detailed assessment with questionnaires, exhaled nitric oxide, spirometry, methacholine, mannitol, EVH, exercise, induced sputum cell count and sputum and urine mast cell mediator measurement.

All athletes with significant airways eosinophilia ($>3\%$) had positive tests to methacholine and EVH. The % fall in FEV₁ post EVH had the strongest correlation with sputum eosinophilia (% fall FEV₁ EVH v logeos: $r=0.652$, $p=0.005$) with an optimum cut point of $\geq 25\%$ (sens: 83.33%, spec: 90.91%). There was a strong correlation between exhaled nitric oxide and sputum eosinophilia (log FE_{NO} v logeos: $r=0.577$, $p=0.015$), best cut off was FE_{NO} of > 45 ppb (sens: 66.67%, spec: 89.94%). All indirect challenges were associated with significantly higher airways PGE₂ (ANOVA, $r^2=0.646$, $p<0.0001$). There was no difference in mast cell mediator concentrations in sputum or urine between the testing modalities (ANOVA $r^2= 0.026$, $p=0.644$).

In this cross-sectional study of 17 athletes the bronchoconstrictor response to either direct or indirect challenge tests varies widely and does not relate particularly closely to each other, underlying eosinophilic airways inflammation or markers of mast cell activation.

Introduction

There is a high prevalence of exercise induced bronchoconstriction in elite performance athletes, when compared to the general population (21). Because of concerns about abuse of beta agonists, sporting regulatory bodies advocate the use of objective challenge tests to determine who should compete on anti-asthma medication in elite sport (25). A number of challenge tests are permitted under current anti-doping rules including inhaled histamine and methacholine, which act directly on airway smooth muscle; and indirect challenge tests such as exercise, eucapnic voluntary hyperpnoea and mannitol, which involve an intermediary pathway such as mast cells or neuronal pathways and thus may provide a more complete assessment of the asthmatic process (120).

In asthma there is evidence that the response to indirect bronchoconstrictor challenges is associated with eosinophilic airway inflammation and mast cell mediator release. This might not be the case in elite endurance athletes as the pathophysiology of airways hyperreactivity (58) may be more closely linked to mast cell activation than airway eosinophilia (97;127). However, how responses to these various tests relate to the patterns of airways inflammatory mediator release or to the cellular pattern of airways inflammation in elite endurance athletes remains to be fully determined.

We set out to investigate how responses to standardized direct and indirect challenge tests relate to underlying patterns of airways inflammation and airway mediator release in a cohort of international level endurance athletes who reported symptoms suggesting exercise-induced asthma. We sought to determine the relationship between symptoms, airways dysfunction, mast cell mediator release and cellular airways inflammatory patterns with the ultimate aims of furthering our understanding of the underlying pathophysiological mechanisms and determining which challenge test, amongst those recommended for elite athletes, would best identify clinically significant pathology.

Methods:

Subjects

Seventeen endurance athletes, all exercising for more than 15 hours per week, from a variety of sporting backgrounds agreed to take part in the trial. All were competing at international level and had expressed symptoms suggestive of exercise asthma and had been previously using medication whilst competing under a therapeutic use exemption (under the then current guidance). They were all either inhaled corticosteroid naïve or had withdrawn inhaled corticosteroids and long acting beta-agonists for at least 4 weeks prior to starting the study. All challenge tests were carried out at least 48 hours apart and following an 8 hour interval from previous exercise, short acting beta-agonist use or caffeine.

Study design

The study was performed at the Respiratory Research Unit, Institute for Lung Health, Glenfield Hospital, Leicester and at the Department for Human Sciences, University of Loughborough, Loughborough, UK. Participants visited the research units on four separate occasions. An initial assessment visit included a physical examination, detailed medical

history questionnaire, Juniper asthma control questionnaire (ACQ), Asthma Quality of Life Questionnaire (AQLQ), exhaled nitric oxide (FE_{NO}) measured using the Niox MINO, baseline spirometry, a methacholine PC₂₀, skin prick tests against common aeroallergens (dog, cat, HDM, grasses, tree, aspergillus, histamine control) and an induced sputum differential inflammatory cell count was obtained on each occasion. Visit 2 entailed measurement of FE_{NO}, spirometry and a eucapnic voluntary hyperpnoea (EVH) provocation test. Visit 3 measured FE_{NO}, spirometry and a mannitol provocation test. Visit 4 measured FE_{NO}, spirometry and an exercise challenge test. In order to avoid any effect of bronchial provocation with one agent on the outcome of provocation with another agent, there was an interval of at least 48 h between challenges. The study was approved by the local regional ethics committee (Study UHL 10589); all gave written informed consent.

Measurements

Single flow FE_{NO}, spirometry and questionnaire scores were recorded at 50 ml/s as previously described in the methods section.

Challenge tests:

Methacholine PC₂₀: The methacholine PC₂₀ was measured using the standard tidal breathing method with a maximum inhaled concentration of 16 mg/ml, as previously described in the methods section.

Mannitol: Bronchial provocation testing was done using the standard method.

Eucapnic voluntary hyperventilation: Bronchial provocation challenge testing with eucapnic voluntary hyperventilation (EVH) was then performed as per previously described standards (120).

Urine: The athletes were encouraged to drink freely before the test and afterwards during recovery. At 60 minutes post the start of the EVH test and prior to sputum induction, a urine specimen was attained for mediator metabolite assay.

Sputum supernatants were prepared as described and sputum mediator concentrations were measured using commercially available enzyme linked immune-sorbent assays according to the manufacturers agreed protocols.

Statistical methods

Graph Pad Prism (version 5) and SPSS version 14 were used for data analysis.

Results:

Seventeen international endurance athletes consented to take part in the trial and all of these underwent all four challenge tests and were able to produce a sputum and urine specimen suitable for analysis on each occasion. Their baseline characteristics are summarized in Table 1. There were 7 male and 10 female athletes with 9 swimmers and 8 non-pool athletes. The mean (range) age was 23 years (range 19-29 years) and mean (SD) FEV₁ % predicted was 110.6% (SD 12.03). Nine athletes (53%) had one or more positive skin prick tests and 8 (47%; 7 with positive skin tests) had a history of asthma in childhood. These were the first 17

volunteers and had not been pre-selected in anyway and are felt to be representative of the population as a whole.

The results of the various challenge tests are given in table 2. All athletes with a history of childhood asthma had a positive result to methacholine, and to EVH, whereas only 6/9 (67%) had a positive test to mannitol and only 5/9 (56%) to exercise. There was no correlation between symptom scores and a positive result to any of the testing modalities.

No.	sex	age	FEV ₁ % pred	sport	ACQ	AQLQ	Atopy	Child asthma	FeNO (ppb)	Eosi %	Neutros %	Epithel %
1	F	25.	106.00	Rowing	0.28	6.28	Y	Y	75.	5.50	33.75	0.75
2	F	20.	123.00	Track	1.00	5.94	Y	N	20.	0.00	78.00	0.75
3	F	19.	124.00	Swim	0.71	6.72	N	Y	10.	0.25	4.75	88.75
4	F	27.	101.00	Track	1.14	5.59	N	N	31.	0.25	79.25	0.75
5	F	24.	120.00	Swim	0.29	6.84	N	N	30.	0.25	63.50	3.50
6	F	19.	116.00	Swim	0.86	5.72	Y	N	44.	4.00	77.75	1.50
7	M	21.	105.00	Swim	2.14	3.59	Y	Y	84.	4.25	79.25	5.75
8	F	23.	122.00	Swim	0.71	6.25	N	N	16.	0.00	56.75	7.50
9	F	19.	118.00	Swim	0.71	6.25	N	N	9.	0.75	94.00	0.00
10	M	25.	104.00	Swim	0.00	6.84	N	N	25.	0.00	53.75	2.00
11	M	23.	134.00	Swim	0.71	6.03	Y	Y	79.	2.25	5.50	11.00
12	M	21.	109.00	Swim	0.14	6.69	Y	Y	72.	5.00	77.00	9.00
13	M	19.	97.60	Track	0.29	6.22	Y	Y	24.	4.00	91.75	3.00
14	F	20.	113.00	Track	0.57	6.28	N	N	14.	0.00	57.50	2.00
15	M	25.	99.00	Rower	0.43	6.72	Y	Y	44.	9.25	5.25	92.50
16	F	29.	102.00	Track	1.42	4.78	Y	Y	46.	0.00	16.75	0.75
17	M	26.	87.00	Tri	1.71	5.50	N	N	26.	1.25	92.00	0.25

Table 1: Athletes baseline characteristics and airways cellular inflammation profiles. ACQ is a 7 item questionnaire on a 7 point scale looking at asthma control in the previous week. Scores are given as the mean of the 7 answers, lower scores indicating better control. The AQLQ is a 32 item questionnaire rated on a 7 point scale to yield a mean overall score out of seven. The higher the score the better quality of life.

Relationship between test results and the induced sputum eosinophil count

All those athletes with significant eosinophilic airways disease (>3%) had positive tests to methacholine and to the EVH test. The % fall in FEV₁ post challenge on EVH had the strongest correlation with sputum eosinophil % (% fall FEV₁ EVH v logeos: r=0.652, p=0.005). The standard cut point (>10% fall in FEV₁ at two time points post challenge) was very sensitive (100%), but not particularly specific (45%) for determining a clinically significant degree of eosinophilic airways inflammation (sputum eosinophil count >3%). The optimum cut point to determine a significant degree of eosinophilic airways disease was a drop post challenge of ≥25% (sens: 83.33%, spec: 90.91%), fig 1. There was a strong correlation between exhaled nitric oxide and sputum eosinophilia (log FE_{NO} v logeos: r=0.577, p=0.015). The best cut off for eosinophilic disease was a FE_{NO} of > 45 ppb (sens: 66.67%, spec: 89.94%). A composite score of a 10% fall in FEV₁ post EVH challenge and a FE_{NO} >45 ppb had a sensitivity of 75% and a specificity of 91% for the presence of a sputum eosinophil count >3%. FE_{NO} also proved to be a consistent marker, with an intra-class correlation coefficient of 0.959 (CI 0.919 – 0.982, F= 26.223, df1=19, df2=57, p<0.0001). The eosinophilic phenotype was also stable; within subject ICC 0.94 (CI 0.882 – 0.969, F=58.579, df1=22, df2=66, p<0.0001), meaning those who were eosinophilic stayed eosinophilic throughout.

Relationship between test results and sputum markers of mast cell activation

Sputum mediator concentrations after challenge are shown in Fig 2. We found no evidence that mast cell derived mediators were present in higher concentrations after indirect challenge compared to methacholine. All the indirect challenge tests were associated with significantly higher luminal concentrations of PGE₂ (ANOVA, r²=0.646, p<0.0001).

We considered whether these results reflected differences in the level or degree of bronchoconstriction induced and, therefore, corrected the concentration of mediator for the % fall in FEV₁ post challenge. This analysis showed mannitol to be a potent stimulator of histamine release irrespective of whether it causes bronchoconstriction (ANOVA, histamine p<0.0001, PGD2 p=0.293, cyst-I_t p=0.18) (Fig 4). Even correcting for % fall in FEV₁, the indirect challenges were associated with higher concentrations of sputum PGE2 (ANOVA, PGE2 p=0.008).

We looked at systemic markers of mast cell activation by measuring the urinary concentration of the PGD2 metabolite 9 α -11 β -PGF2 in the urine specimens attained 60 minutes post airways challenge (Fig 5). There was no difference in the amount of urinary mediator concentration between the different testing modalities when corrected for creatinine concentration (Fig 5a) (ANOVA r²= 0.026, p=0.644). Even when corrected for change from methacholine (ANOVA p=0.06) and % fall in FEV₁ (ANOVA p=0.54), indirect tests were associated with similar levels of mediator concentration to those detected after methacholine.

Relationship between the response to different challenges

There was a particularly strong correlation between a positive result to methacholine and a positive result to EVH (r=0.859, p=0.00001) but not between methacholine and either mannitol or exercise, nor between the indirect challenge methods with each other. The relationship between methacholine and mannitol responsiveness and the other tests was not appreciably altered when the results of the challenge test were expressed as the value obtained from the dose-response slope (table 3).

Table 2: Heterogenous responses to the various challenge tests. We took the standard clinical cut points to determine a positive or negative test. These were a PC₂₀ of <4mg/ml for methacholine; a fall in FEV₁ of >10% at two time points more than 5 minutes apart for EVH; a mannitol PD₁₅ of <635mg; and a drop of >10% FEV₁ post exercise challenge. A negative methacholine has been given the nominative value of 16mg/ml and a negative mannitol a nominative PD15 of 650mg.

Number	Methacholine PC20 mg/ml	Post EVH % fall in FEV ₁	Post Exercise % fall in FEV ₁	Mannitol PC15 mg
1	2.90	42.00	18.00	119.23
2	16.00	6.00	13.00	650.00
3	0.40	19.00	13.00	650.00
4	16.00	9.00	14.00	650.00
5	0.50	19.00	11.00	650.00
6	0.60	39.00	16.00	650.00
7	0.10	53.00	20.00	31.27
8	0.40	10.00	3.00	143.79
9	2.00	11.50	6.00	650.00
10	16.00	22.00	3.00	650.00
11	2.00	29.00	2.00	124.79
12	4.00	43.00	6.00	248.42
13	1.70	12.00	4.50	650.00
14	16.00	4.00	4.00	650.00
15	3.90	28.00	14.00	470.45
16	3.20	17.00	0.00	650.00
17	16.00	6.00	5.00	650.00

Table 3: Correlation matrix of the various challenge test results (r value (p=)).

	Meth PC20	Mann PD15	Exercise	EVH
Meth PC20	1	0.351 (0.130)	-0.306 (0.388)	-0.232 (0.166)
Mann PD15	0.351 (0.130)	1	-0.147 (0.59)	-0.645 (0.004)
Exercise	-0.306 (0.388)	-0.147 (0.59)	1	0.495 (0.051)
EVH	-0.232 (0.166)	-0.645 (0.004)	0.495 (0.051)	1

Discussion:

In this cross-sectional study of 17 elite athletes we have shown that the bronchoconstrictor response to either direct or indirect challenge tests varies widely and does not relate particularly closely to underlying eosinophilic airways inflammation, markers of mast cell activation or each other. Our findings did not support the widely held view that indirect challenges involve common pathways which relate more closely to the presence of asthmatic type airway inflammation. In particular, there was poor correlation between the response on exercise challenge and response to the other testing modalities used. The strongest agreement was between the response to inhaled methacholine and that to EVH, and both correlated closely to a history of childhood asthma, but not atopy. However, on the basis of our findings it would be difficult to make a strong case that this was the case for other challenges.

The challenge test which was most closely related to a significant sputum eosinophilia was the response to the EVH challenge test, which remains the World Anti-Doping Association (WADA) preferred test. The currently advocated criterion for a positive response to EVH (a drop in FEV₁ of 10% or more) is a sensitive but not specific marker of eosinophilic airway inflammation. However, in this group of athletes the best single test to identify significant eosinophilic airways disease was FE_{NO}, with an FE_{NO} >45 ppb giving the best prediction of clinically significant sputum eosinophilia. This cut point is similar to that which has been shown to best identify corticosteroid responsive disease in non-athletic asthma patients (87). Our findings are consistent with the view that airway hyperresponsiveness and eosinophilic airway inflammation are separate, relatively independent domains of asthma (9). They also

suggest that FE_{NO} might be a simple and non-invasive method to determine this characteristic in athletes.

Previous work has shown within subject heterogeneity of response to the various indirect bronchial challenge tests in athletes with symptoms suggestive of exercise induced bronchoconstriction (64;97). Our findings extend this earlier work by allowing us to relate the response to the different challenges to the degree of eosinophilic airways inflammation and mast cell mediator release. They contrast to studies in asthma in the non-athletic population, which have shown that the different indirect bronchoconstrictor challenges investigated in our study relate closely to each other, to eosinophilic airway inflammation and to mast cell mediator release (38;59;120). There are several potential explanations for this. Firstly, exercise induced bronchoconstriction in athletes is often experienced in the absence of other symptoms of asthma and may have a different mechanism and relationship with non-specific airway responsiveness and eosinophilic airway inflammation (24). Secondly this relationship may differ across sports because of sport specific heterogeneity of airway inflammation and dysfunction (40). Our study was not adequately powered to evaluate this definitively; further studies are required to investigate this possibility. Thirdly, exercise challenge may be less sensitive in athletes because of increased cardiovascular fitness and difficulty reproducing the unique environmental conditions responsible for the response. It is possible that field based exercise-challenges would be better, but how these relate to other challenge tests remains to be fully evaluated (64). Fourthly, the method of challenge may be critical. Challenge tests that use incremental doses rather than an all or nothing discrete challenge have the disadvantage of providing no discrimination between patients that test normal. However, when we expressed these results as extrapolations from the dose response curve it did not appreciably alter our findings. Tests that use progressive doses may also induce refractoriness

during the challenge altering the response to the test and increasing variability between tests. Whilst refractoriness to indirect challenge is a well described phenomenon in mild asthma the prevalence of this in elite athletes and its effects on training and competition performance have not been fully investigated. Finally, there may be increased measurement error in an elite athlete population because of variation in airway inflammation and dysfunction coinciding with the training cycle, tapering for major events or out of season recovery periods. In this study there was excellent inter-visit correlation in measurements of airways inflammation (FE_{NO} and sputum eosinophil %) suggesting that steroid naïve individuals have consistent levels of background eosinophilic disease. However, we have no corresponding data on the stability of airways dysfunction over the same training cycle.

In recent years there has been considerable interest in the role of the mast cell in EIB and in the determination of AHR in athletes (97). The levels of mast cell mediators measured in both the sputum and the urine after various indirect challenge tests have been shown to correlate to the degree of bronchoconstriction (105;107;129) and are reduced when bronchoconstriction is attenuated (108;109;130). However, none of these studies controlled for the actual bronchoconstriction process by including a post-methacholine control (131). We had hypothesized that if the indirect challenge method relied exclusively on mast cell mediator release then the measured levels of mediators in the sputum and urine following an indirect challenge test would be significantly greater than those measured in the airways following a direct challenge such as methacholine. One difficulty interpreting our results is that we were restricted to assessing inflammation and mediator release after challenge because a pre-challenge sputum induction might have affected subsequent airway responsiveness and airway inflammation. In retrospect a no challenge control day might have allowed us to interpret our data more clearly. However, the implication of our findings is that mediator

release into the airway lumen and serosa either doesn't occur within the limits of detection or reflects bronchoconstriction and is not specific to indirect challenges. Recent work showing that airway remodeling can be induced by repetitive direct airways challenges in the absence of inflammation (101) and it is interesting to speculate that this process may reflect the effect of bronchconstriction on epithelial cell function and mast cell behavior.

We assessed mast cell activation using sputum and urine as these techniques may assess release in different compartments of the airway, with sputum reflecting luminal release, and urine serosal release. In addition, we were keen to assess urinary markers as these have been found to be elevated more consistently after indirect challenge than sputum markers and may be less affected by the transient nature of histamine in the airway. We measured the PGD2 metabolite 9α - 11β -PGF2 in the urine between 30-90 minutes post challenge as this marker reflects airway PGD2 activity and previous studies have shown the assay to be reliable and consistent (107;132) although more recent work has shown no differences in levels between patients with asthma of differing severity (133). We collected the urine specimen prior to the sputum induction procedure to make sure it could not affect the results. We have previously shown that sputum mast cell mediator levels measured using the same assays effectively discriminate different asthma and cough phenotypes (87;134) and allow demonstration of mediator release after allergen challenge (135).

We looked at a number of sputum mediators reflecting mast cell activation and airway inflammation more generally (87;136). The most striking and consistent result was the significant degree of increased PGE2 measured in the airway following all of the indirect challenges when compared to the direct challenge. PGE2 may have a bronchoprotective role

(111) in the airways and is thought to be responsible for the refractory period commonly seen after indirect bronchoconstrictor challenges (137). Refractoriness does not occur after methacholine challenge in patients with asthma. Our findings suggest that this is because the challenge does not stimulate airway PGE₂ production. Indirect challenge methods may induce more release as they impart greater stress to the airway epithelium (58). There was significantly less histamine in the airway lumen following EVH challenge testing when compared to the other challenge methods. This may reflect high ventilation rates required for this test and increased ‘wash out’ of histamine from the upper airways.

In conclusion, we have shown that the relationship between direct and indirect challenge tests and asthmatic airway inflammation in elite athletes is complex. The complex nature of AHR in athletes, its relationship to eosinophilic airways inflammation, mast cell function and the interaction between the airway epithelium and downstream effector cells remains to be fully elucidated and requires more detailed investigation.

Figures:

Figure 1: ROC curve of the different testing modalities to determine a clinically significant sputum eosinophilia (>3%).

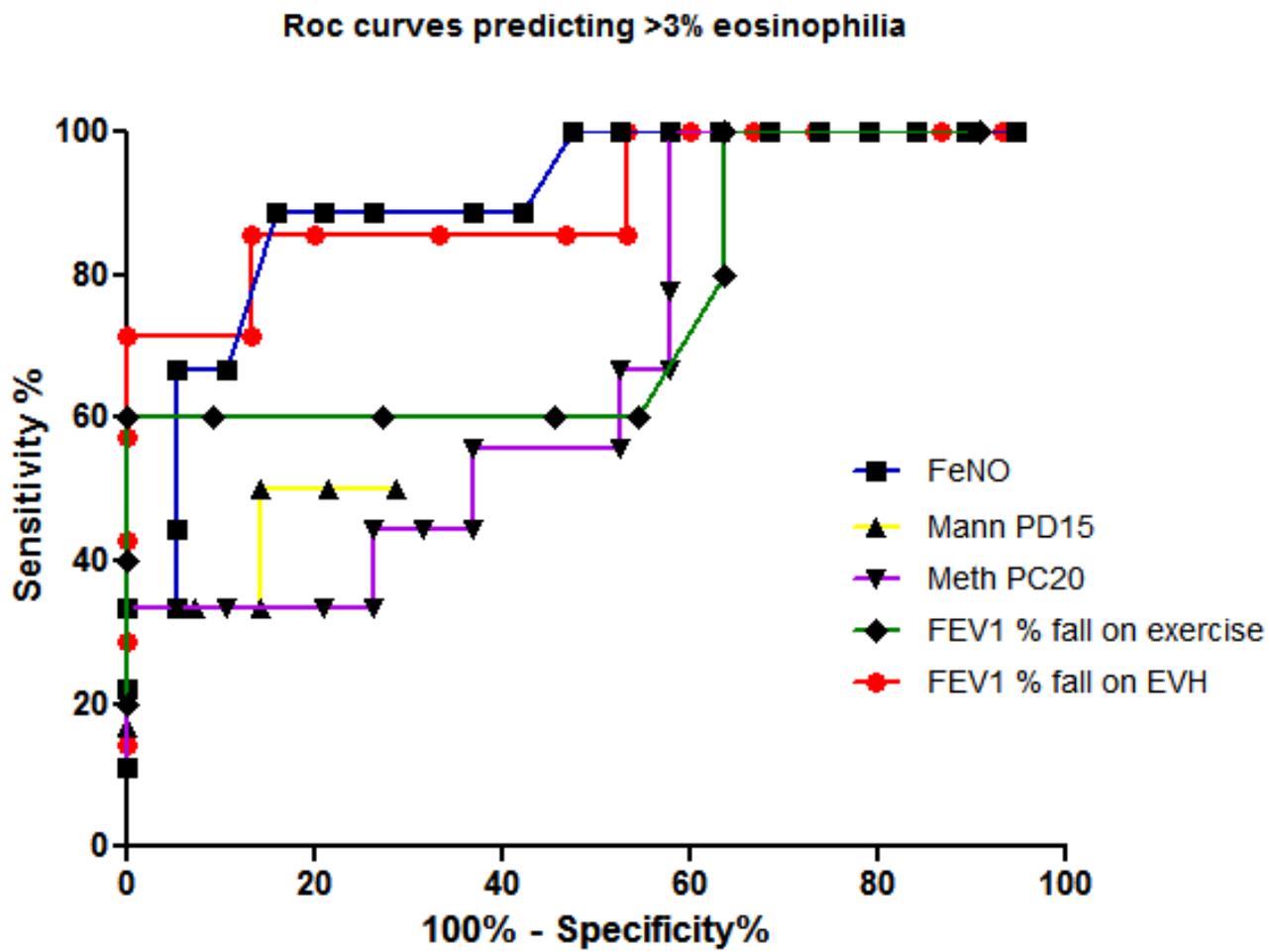
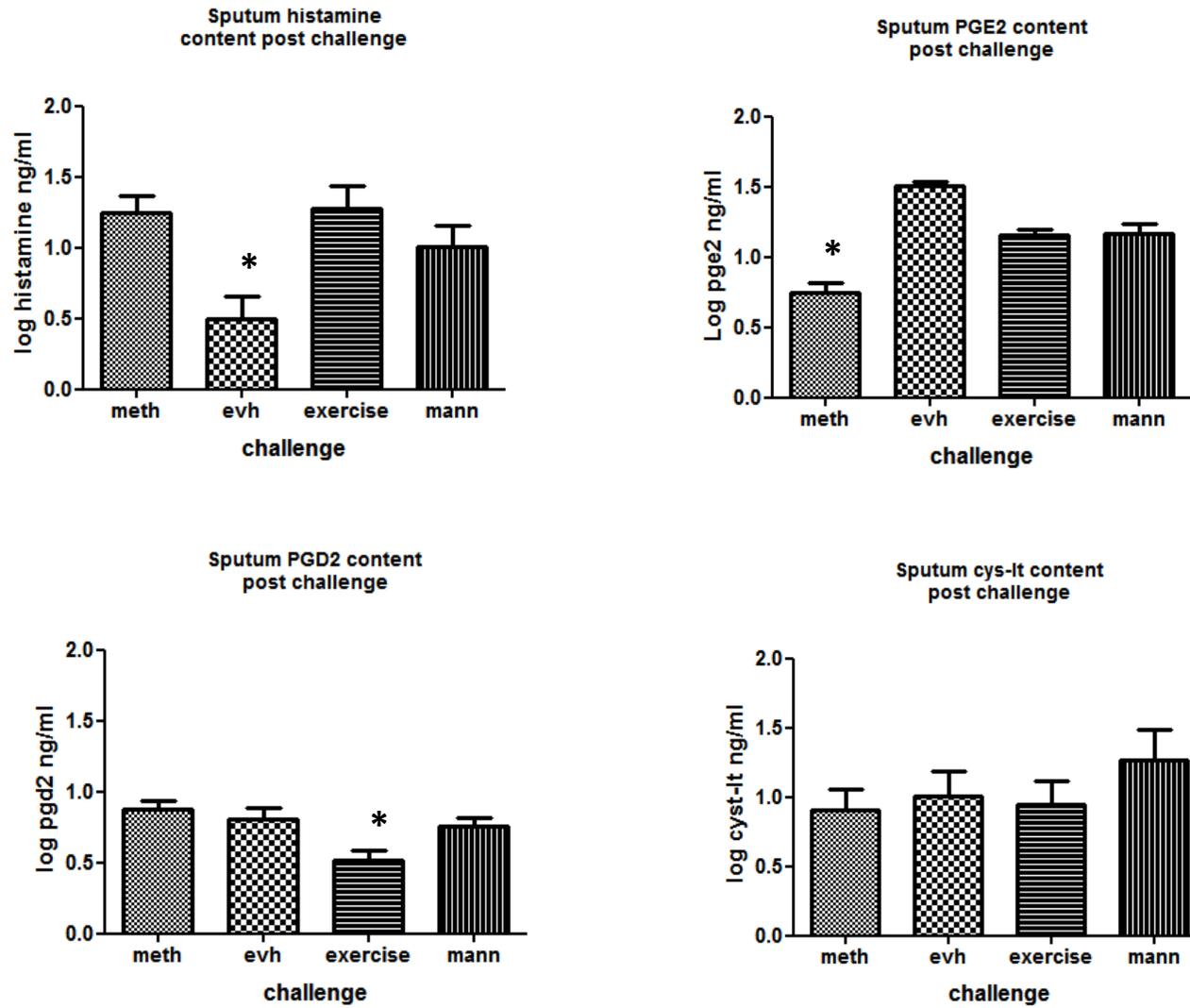
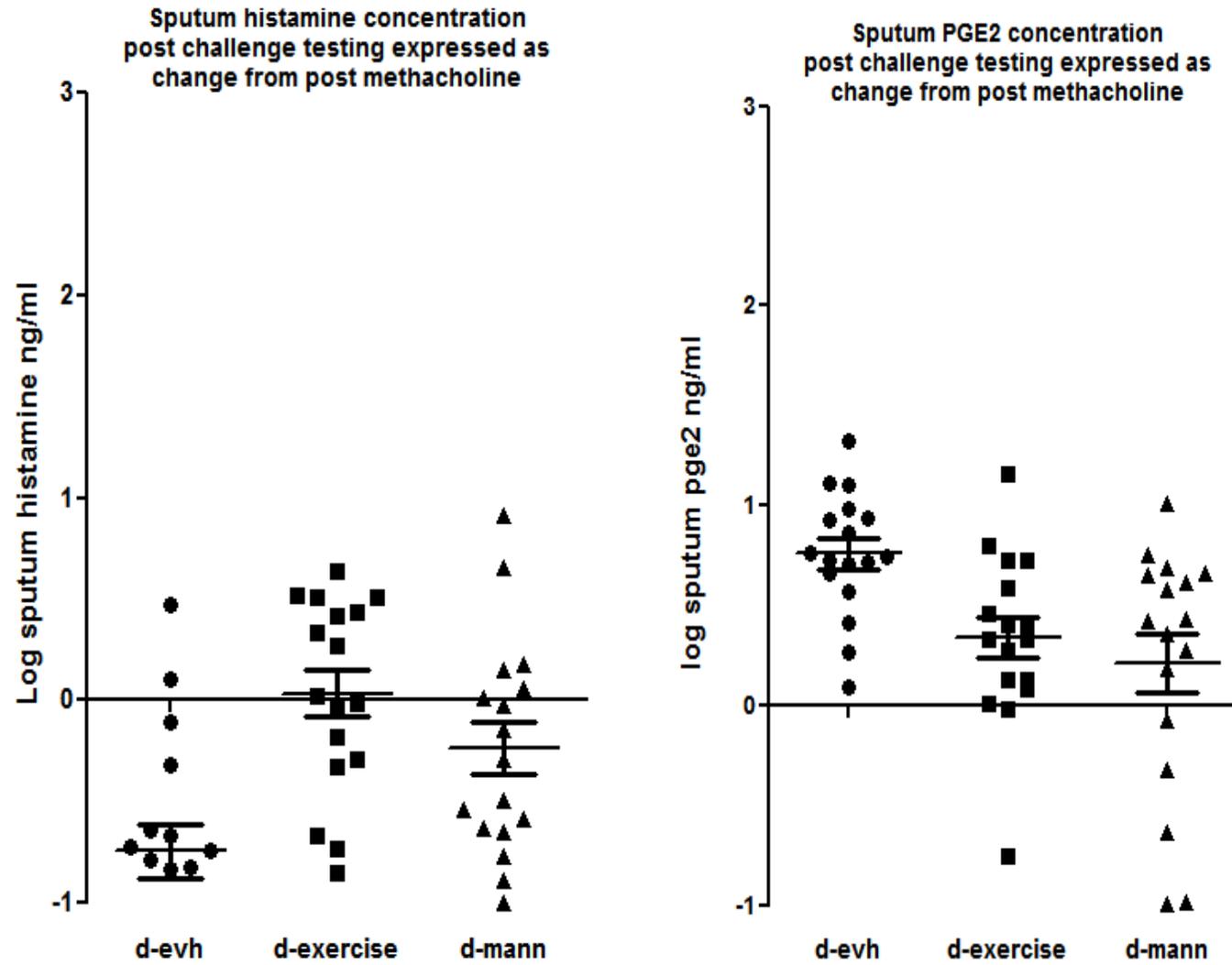


Fig 2: Sputum mediator concentration post direct and indirect airways challenge testing geometric mean \pm SEM ng/m.



*statistically significant ANOVA, p < 0.05

Fig 3: Sputum mediator release corrected for change from methacholine given as geometric mean \pm SEM ng/ml.



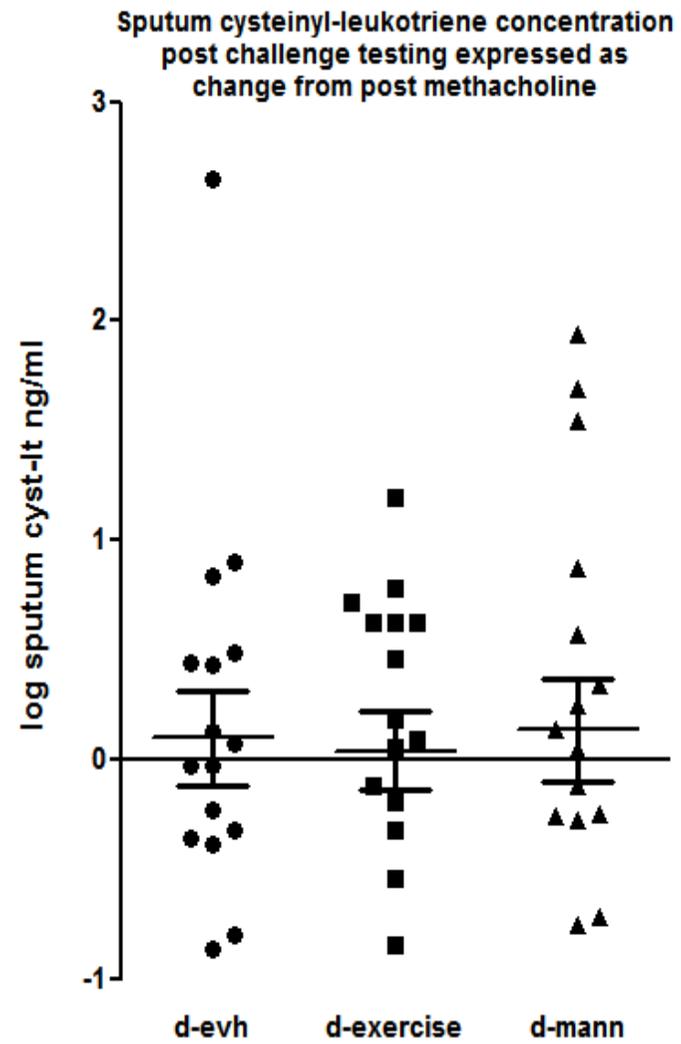
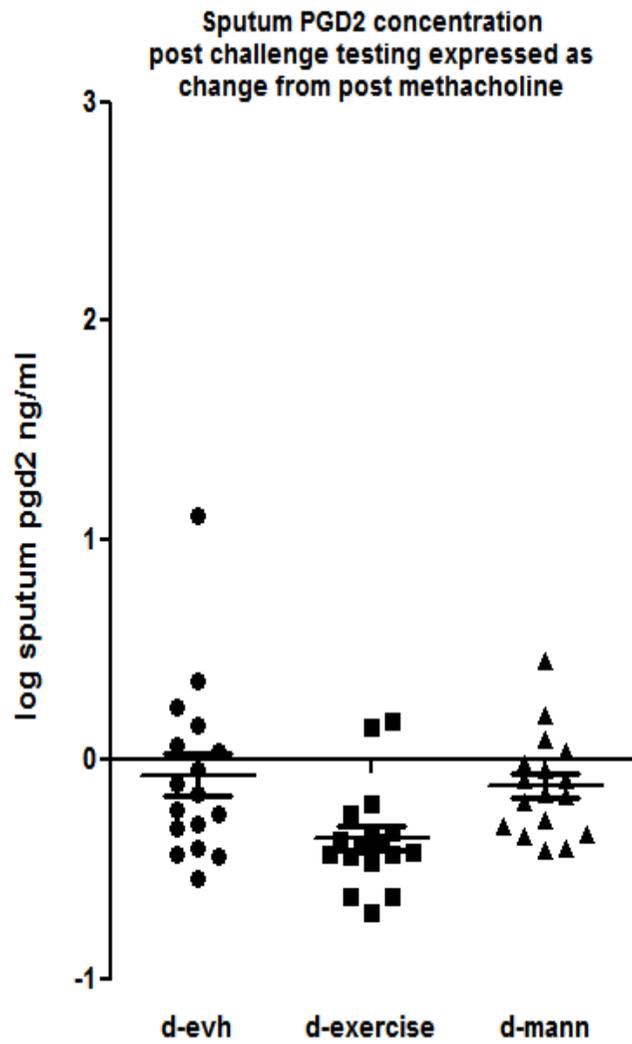


Fig 4: Sputum mediator release corrected for % fall in FEV₁ given as geometric mean \pm SEM ng/ml.

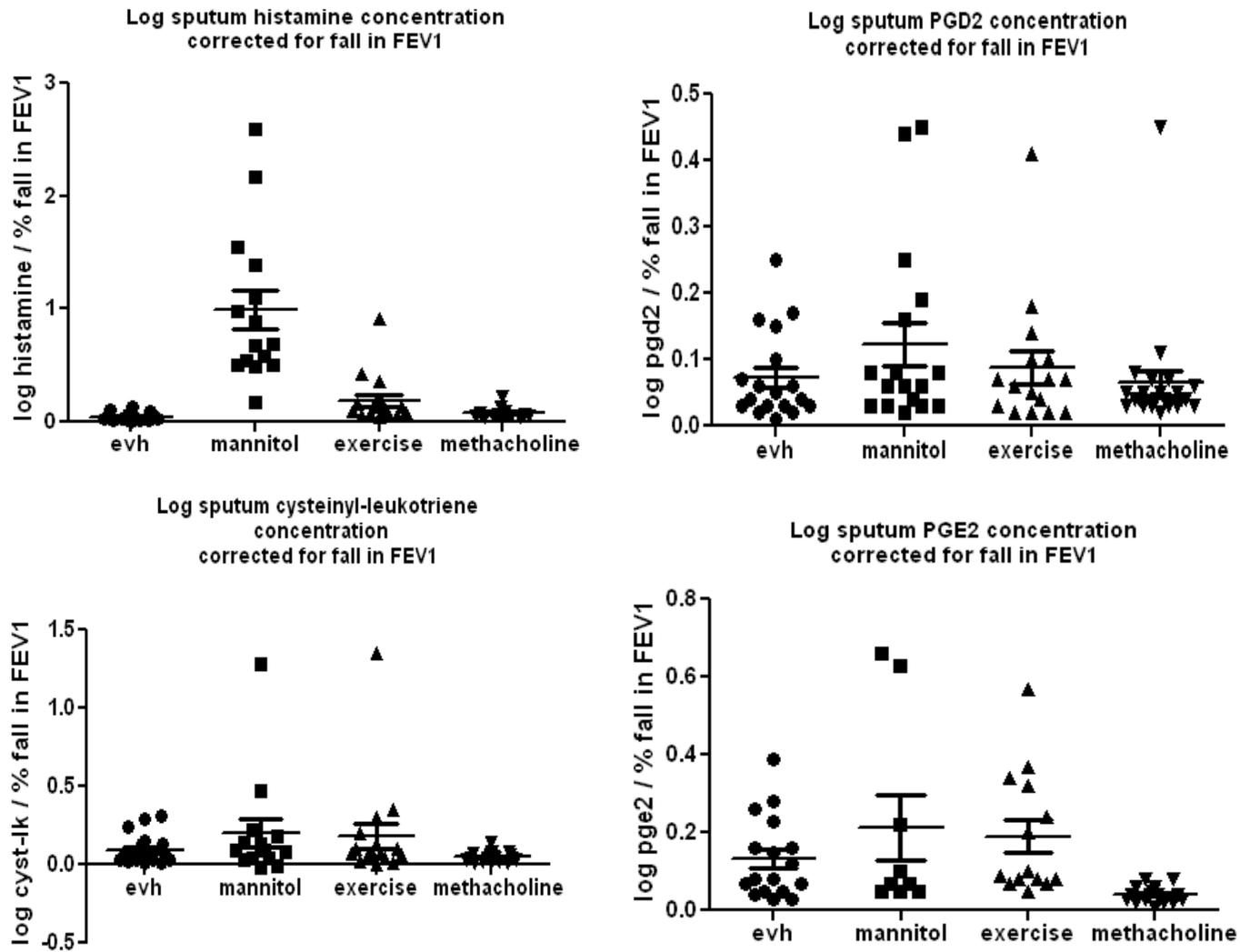
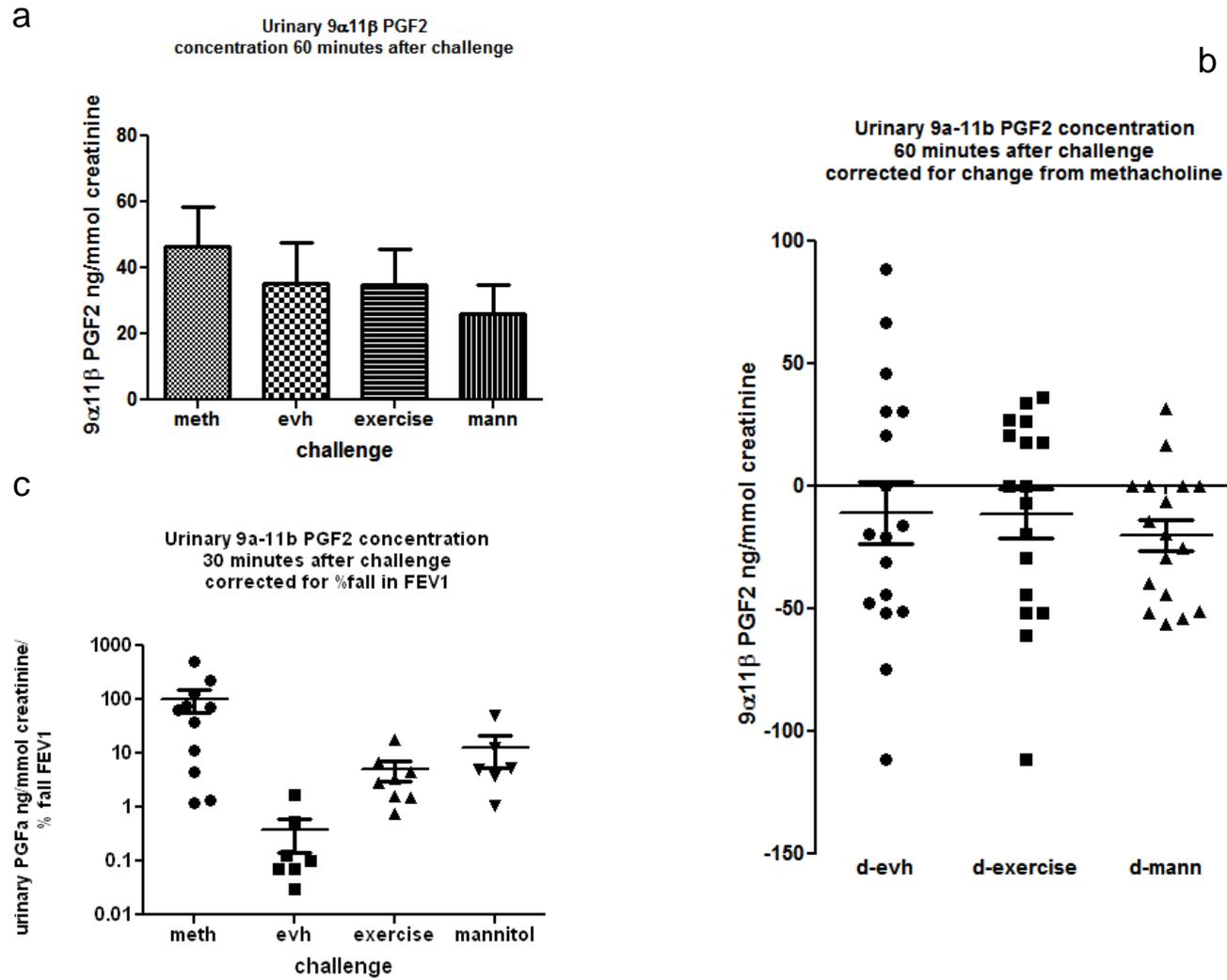


Fig 5: Urinary mediator concentration measured 60 minutes post challenge and corrected for creatinine concentration (a), corrected for % fall in FEV₁ (b), corrected for change from methacholine (c), given as geometric mean \pm SEM ng/ml.



4.2 Airways dysfunction and inflammation in pool and non-pool based elite performance athletes with symptoms suggesting exercise-induced asthma.

Abstract

There is a high prevalence of airways hyperreactivity (AHR) amongst elite athletes. This is particularly high in swimmers, who potentially have higher levels of eosinophilic airways inflammation. We set out to test this hypothesis in a cohort of 118 international athletes.

All subjects had symptoms of exercise asthma and were steroid naïve. They completed baseline spirometry, a symptom score, exhaled nitric oxide (FE_{NO}), an eucapnic voluntary hyperventilation (EVH) test and a post challenge induced sputum and urine test.

Pool based athletes had better lung function (FEV₁ 110 vs 102% predicted; mean difference 8.200 ± 2.339; p=0.0006 and FVC 5.64 vs 4.75 l; mean difference 0.8855 ± 0.1951; p<0.0001) and more marked AHR (% drop in FEV₁ post EVH 18.14 vs 11.47; mean difference 6.67; 95% CI: 2.89 to 10.53; p=0.0009). More pool based athletes had a positive EVH test (72% pool v 39% non-pool), but there was no difference between groups with respect to eosinophilic inflammation (sputum eos % pool=2.07, non-pool=2.28, p=0.77; FE_{NO}, pool = 32.54, non-pool= 35.77, p=0.60). Athletes with a positive EVH test had less neutrophilic inflammation (p=0.01) and more epithelial cells (p=0.03) in their sputum.

Pool based endurance athletes have greater evidence of AHR than non-pool based athletes but no evidence of greater eosinophilic airways inflammation. Athletes who test positive on EVH are more likely to be eosinophilic and have higher levels of epithelial cells in their sputum.

Introduction

It is generally accepted that the prevalence of asthma in the elite sporting population far outstrips that in the general population (20). Questionnaire based studies estimate the prevalence of symptoms suggesting exercise asthma to be up to 30 % in summer athletes and 50% in winter athletes and swimmers (21) against a background population prevalence in adults of about 15%. However, responses on questionnaires alone show poor agreement with objective measures of airway dysfunction (24) and regulatory bodies advocate the use of objective challenge tests to determine who should compete on anti-asthma medication in elite sport (25). The 'gold standard' test used is the Eucapnic Voluntary Hyperventilation test (EVH), designed to mimic the high flow rates achieved by athletes during maximal respiratory effort (64) and using this test it has been demonstrated that there are high levels of airways hyperreactivity in athletes.

Swimmers and winter sport athletes are particularly likely to report symptoms suggesting exercise-induced asthma and to have objective evidence of airway hyperresponsiveness (40;100). One potential explanation is that exercise in a physically challenging and/or polluted environment contributes to airway dysfunction. If this were the case then the pattern of lower airway inflammation might be expected to differ as irritant stimuli and pollution tend to provoke neutrophilic airway inflammation (138). The limited existing data suggests that swimmers have higher levels of eosinophilic airways inflammation (43;100) and that this may be related to the pool environment (139). However, in these studies the swimmers were compared to winter sports athletes who may themselves have a unique, steroid refractory, non-eosinophilic pathology (95). How the levels of inflammation compare to other pool users and summer sports endurance athletes has not been established.

We set out to compare the levels of airways dysfunction and inflammation in a large cohort of elite performance athletes from either a pool or a non-pool summer sports based environment. Our aim was to compare patterns of cellular inflammation and mast cell mediator release in response to the EVH challenge test. We evaluated athletes reporting symptoms suggesting exercise-induced asthma as this is the population who seek medical help and might potentially require treatment, particularly with inhaled corticosteroids. Our aim was to determine the extent of differences in cellular inflammation or mast cell activation in the two groups as this may help us to understand the effect of the training environment and guide us in how best to manage these athletes.

Methods:

Subjects

One hundred and eighteen endurance athletes, exercising for more than 15 hours per week, from a variety of sporting backgrounds agreed to take part in the trial. All were competing at international level and had expressed symptoms suggestive of exercise asthma and would require a therapeutic use exemption and evidence of objective testing to use medication whilst competing. They were a self selected group who presented as patients for assessment. Prior to testing they were all either inhaled corticosteroid naïve or had withdrawn inhaled corticosteroids for at least 4 weeks prior to starting the study. They were classed as pool based athletes if they exercised for more than 5 hours per week in an indoor pool environment and non-pool based athletes if they had less than 0.5 hours per week exposure to an indoor pool environment. The tests were carried out following at least an 8-hour interval from previous exercise, short acting beta agonist use or caffeine.

Study design

The study was performed at the Respiratory Research Unit, Institute for Lung Health, Glenfield Hospital, Leicester and at the Department for Human Sciences, University of Loughborough, Loughborough, UK. An initial assessment included a physical examination, detailed medical history questionnaire, Juniper asthma control questionnaire (ACQ), Asthma Quality of Life Questionnaire (AQLQ), exhaled nitric oxide (FE_{NO}) measured using the Niox MINO, baseline spirometry, and a eucapnic voluntary hyperpnoea (EVH) provocation test. Following the test and recovery a urine specimen was collected for mediator assay and then an induced sputum sample was collected for differential inflammatory cell count. Finally they underwent skin prick tests against common aeroallergens (dog, cat, HDM, grasses, tree, aspergillus, histamine control). The study was approved by the local regional ethics committee (Study UHL 10589); all gave written informed consent.

Measurements

Single flow FE_{NO}, spirometry, and questionnaires were recorded as previously described.

Eucapnic voluntary hyperventilation: Bronchial provocation challenge testing with eucapnic voluntary hyperventilation (EVH) was then performed as per previously described standards (120). Sputum supernatants were prepared as described and sputum mediator concentrations were measured using commercially available enzyme linked immune-sorbent assays according to the manufacturers agreed protocols.

Statistical methods

Graph Pad Prism (version 5) and SPSS version 14 were used for data analysis.

Results:

One hundred and eighteen international endurance athletes consented to take part in the trial. A summary of their baseline characteristics is shown in table 1. Pool based athletes included

swimmers (n=30), water polo players (n=15) and tri-athletes (n=9), whilst non-pool based athletes were a mixture of rowers (n=10), cyclists (n=15), runners (n=20) and footballers (n=19). The groups were equally matched for age, sex, height and weight. Despite this the pool based athletes had significantly higher FEV₁ (110 vs 102% predicted; mean difference 8.200 ± 2.339; p=0.0006) and FVC (5.64 vs 4.75 l; mean difference 0.8855 ± 0.1951; p<0.0001). They were also significantly more symptomatic when compared to non-pool based athletes (ACQ 0.95 vs 0.36, mean difference 0.5927 ± 0.135; p<0.0001; AQLQ 6.02 vs 6.69, mean difference 0.6738 ± 0.1583; p<0.0001). The populations were well matched for atopy and childhood asthma.

Table 1: Baseline characteristics in pool and non-pool based athletes. *Mean (SEM)

ACQ is a 7 item questionnaire on a 7 point scale looking at asthma control in the previous week. Scores are given as the mean of the 7 answers, lower scores indicating better control. The AQLQ is a 32 item questionnaire rated on a 7 point scale to yield a mean overall score out of seven. The higher the score the better quality of life.

	Pool based (n=54)	Non-pool based (n=64)	
Age (yrs)	21 (16-35)	20 (16-32)	Ns
Sex	44% male	47% male	Ns
Height (m)	1.77 (0.01)	1.75 (0.01)	Ns
Weight (kg)	70.95 (1.38)	71.28 (1.59)	Ns
FEV₁	4.46 (0.11)	4.00 (0.09)	p=0.002
FEV₁ % predicted	110 (1.85)	102 (1.47)	p=0.0006
FVC	5.64 (0.15)	4.75 (0.13)	p<0.0001
ACQ score	0.95 (0.13)	0.36 (0.06)	p<0.0001
AQLQ score	6.02 (0.14)	6.69 (0.08)	p<0.0001
Symptom score at rest	9.6 (0.4)	7.8 (0.4)	p=0.004
Symptom score on exercise	13.5 (0.5)	11.6 (0.7)	p=0.03
Childhood asthma	N=23 (42.5%)	N=26 (40%)	Ns
Atopy	12 (22%)	8 (12.5%)	p=0.16

The sputum differential cell counts for the two groups of athletes are shown in Table 2. The geometric mean sputum eosinophil ($p=0.77$), mean sputum neutrophil count ($p=0.24$) and mean sputum epithelial cell count ($p=0.19$) did not differ significantly between groups and there was no significant difference in the geometric mean concentration of exhaled nitric oxide. However, there were significant differences in the response to the EVH test, with significantly more pool based athletes recording a positive test according to IOC-MC recommended cut off points ($>10\%$ drop in FEV_1) (72% pool v 39% non-pool) ($p=0.0009$, $n=118$) (Fig 1) and a greater mean maximum fall post challenge (18.14 vs 11.47; mean difference 6.67; 95% CI: 2.89 to 10.53; $p=0.0009$).

Table 2: Sputum cell count differentials in pool and non-pool based athletes. Mean (SEM)

	Pool based (n=54)	Non-pool based(n=64)	
FE_{NO}	32.54 (4.9)	35.77 (3.76)	Ns
Mean sputum eosinophils %	2.07 (0.54)	2.28 (0.58)	Ns
Mean sputum neutrophil %	51.33 (4.26)	63.8 (4.17)	Ns
Mean sputum epithelial cell %	18.79 (3.47)	11.20 (2.75)	Ns

There was a weak correlation between the presence of symptoms, symptom scores and the % fall in FEV_1 post EVH ($r=0.21$, $p=0.03$), or the degree of eosinophilic airways inflammation ($r=0.251$, $p=0.004$) (Fig 2).

Table 3 shows the baseline characteristics in the populations sub-divided by a positive ($>10\%$ fall in FEV_1) or negative EVH test. Athletes with a positive test had a larger FVC, greater rest

and on exercise symptoms on the athlete asthma questionnaire and significantly more had a history of asthma in childhood.

Table 3: Baseline characteristics of athletes who had either a positive or negative EVH test. *Mean (SEM)

	>10% drop in FEV₁ post EVH (n=64)	<10 % drop in FEV₁post EVH (n=54)	
Age (yrs)	21 (16-35)	20 (16-32)	p=0.07
Sex	50 % male	48 % male	p=0.46
Height (m)	1.76 (0.01)	1.75 (0.01)	p=0.69
Weight (kg)	72.85 (1.56)	70.11 (1.44)	p=0.20
FEV₁	4.27 (0.11)	4.11 (0.17)	p=0.28
FEV₁ % predicted	105 (1.61)	104 (1.96)	p=0.55
FVC	5.41 (0.15)	4.87 (0.13)	p=0.01
ACQ score	0.77 (0.10)	0.60 (0.11)	p=0.28
AQLQ score	6.20 (0.11)	6.41 (0.13)	p=0.22
Symptom score at rest	9.6 (0.41)	7.9 (0.42)	p=0.007
Symptom score exercise	13.8 (0.54)	11.3 (0.69)	p=0.005
Childhood asthma	N=37 (58%)	N=15 (28%)	p=0.0002
Atopy	15 (23%)	10 (18.5%)	p=0.35

There were significant differences in the sputum differential cell counts between those athletes that had a positive test and those that had a negative test (table 4). In particular, there was significantly greater levels of eosinophilic airways inflammation in those who had a positive EVH test (log sputum eos % pos v neg, p=0.0009) and this was mirrored in the levels of measured exhaled nitric oxide (log sputum FE_{NO} pos v neg, p=0.008). There was also significantly less neutrophilic airway inflammation in those with a positive test (log sputum neutros pos v neg, p=0.01) and significantly more epithelial cells in the sputum (log sputum epithelial % pos v neg, p=0.03).

Table 4: Sputum cell count differentials in those athletes with a positive or negative EVH test. Mean (SEM)

	Positive EVH	Negative EVH	
	(n=64)	(n=54)	
FE_{NO}	43.80 (5.10)	26.78 (3.50)	p=0.008
Mean sputum eosinophils %	3.35 (0.94)	1.43 (0.26)	p=0.0009
Mean sputum neutrophil %	50.83 (3.99)	65.22 (4.02)	p=0.01
Mean sputum epithelial cell %	18.74 (3.40)	9.42 (2.24)	p=0.03

There was no difference between pool and non-pool based athletes in sputum histamine (log sputum histamine pool v non-pool, p=0.74), PGD2 (log sputum PGD2 pool v non-pool, p=0.49), or cysteinyl leukotriene concentrations (log sputum cyst-lt pool v non-pool, p=0.09). There was significantly more PGE2 in the airways of pool based athletes compared to non-pool athletes (p=0.01) (Fig 3). There was no difference in the mean concentration of urine PGF2 pool v non-pool, p=0.10). Neither did mast cell mediator concentrations in either the sputum or the urine differ appreciably between those who had a positive or a negative test (log sputum histamine pos v neg, p=0.40; log sputum PGD2, p=0.84; log sputum cyst-lt, p=0.86; log sputum PGE2, p=0.08; urinary PGF2, p=0.08) (Fig 4).

Across the population as a whole there was good correlation between the degree of eosinophilic airways inflammation and FE_{NO}. The optimum cut point was an FE_{NO} of 43ppb giving a sensitivity of 80% and specificity of 91% for a sputum eosinophil count of >3% (ROC: AUC 0.86, p<0.0001).

Discussion:

In this cross-sectional study of elite performance athletes reporting symptoms suggesting exercise-induced asthma, we have shown that there is no significant difference in the pattern of airways inflammation between pool and non-pool based elite performance athletes. However, pool based athletes are more symptomatic, have higher FEV₁ and FVC and are more likely to demonstrate AHR. This is the first time that such a large group of swimmers has been directly compared to summer sports athletes as opposed to the more select and defined population of winter sports athletes.

Pool based athletes were more 'asthmatic' than their non-pool based counterparts (they were more symptomatic and more likely to have a significant degree of AHR), despite having greater resting lung function. This increased lung size may have played a part in this by generating larger ventilation excursions during EVH testing, giving the airway mucosa a greater challenge. It may also be possible that the increased vital capacity and air trapping associated with asthma has a performance advantage for swimmers as larger lung volumes leads to increased buoyancy and reduced aquatic drag coefficients (141). The increased levels of PGE₂ measured in the airways of pool based athletes may be an important marker of airways stress. PGE₂ is thought to have a bronchoprotective role in the human airway (111) and it may be raised in response to an external stressor such as chemical products in the water or increased osmolar stress related to the ventilator challenge. This again may be related to the larger resting lung function seen in the pool based group.

It has been proposed that swimmers have a higher degree of eosinophilic airway inflammation than non-swimmers (43;100) perhaps reflecting airways sensitization to pool based chemicals (139). However, in these cohorts swimmers were compared directly against winter sports athletes who themselves should be considered a specific phenotype for airways

inflammation (95). When compared to non-pool based athletes participating in endurance sports at a similar level, we found no difference in the pattern of cellular airways inflammation between the two groups. Our findings do not support the view that swimmers have a specific pattern of airway inflammation linked to exposure to pool based sensitisers or irritants. The study was large and adequately powered to exclude a 2 fold difference in sputum eosinophils and a 13% increase in sputum neutrophils, values that have previously been defined as clinically significant (10).

We assessed mast cell activation using sputum and urine as these techniques may assess release in different compartments of the airway, with sputum reflecting luminal release, and urine serosal release. In addition, we were keen to assess urinary markers as these have been found to be elevated more consistently after indirect challenge than sputum markers (121) and may be less affected by the transient nature of histamine in the airway. We measured the PGD2 metabolite 9α - 11β -PGF2 in the urine between 30-90 minutes post challenge as this marker reflects airway PGD2 activity and previous studies have shown the assay to be reliable and consistent (107;129) although more recent work has shown no differences in levels between patients with asthma of differing severity (133). Sputum mast cell mediator levels measured using the same assays effectively discriminate different asthma and cough phenotypes (87;134) and allow demonstration of mediator release after allergen challenge (135). We collected the urine specimen prior to the sputum induction procedure to make sure it could not affect the results.

Eosinophilic airways inflammation was not a universal finding in elite performance athletes but its presence did tend to track with other indicators of asthma. In keeping with our earlier findings in a smaller population, we found a weak correlation between the sputum

eosinophils and the response to EVH, implying that these tests assess different aspects of the disease and potentially relate to symptoms and other aspects of the disease differently. The lack of any clear differences in mast cell mediator levels between the athletes who have had a positive test when compared to those with a limited response to the stimulus is also consistent with our earlier work. This raises questions about the significance of these mediators

This large cohort of athletes also allowed us to look in more detail at the airways inflammatory markers associated with having a positive test result to the EVH indirect challenge test. Most notably there was a higher proportion of eosinophilic athletes who had a positive test (30%) compared to those who had a negative test (4%) when using a standard cut point of $>3\%$ for sputum eosinophilia. This was reflected in the significantly different levels of measured FE_{NO} between the groups. There were also significantly more epithelial cells measured in the sputum from those athletes that had a positive test, and significantly less neutrophilic inflammation. In terms of mast cell mediator concentrations in the sputum there was no significant differences between those who had a positive or a negative challenge test result. This was true for both measured mediators in the sputum and metabolites in the urine.

Conclusion

We conclude that pool based endurance athletes have greater evidence of AHR than non-pool based athletes but no evidence of greater levels of eosinophilic airways inflammation. Athletes with a positive EVH test are more likely to have higher levels of eosinophilic airways inflammation and higher levels of epithelial cells in their sputum suggesting damage to the airway lining. There were no differences between the groups with respect to measured levels of mast cell mediators or their metabolites. The detailed pathophysiology of increased

AHR in athletes requires further examination. In particular, the role of the airways epithelium and the bronchoprotective role of PGE₂ need to be investigated in more detail.

Figures:

Fig 1: Maximum sustained measured % drop in FEV₁ post EVH challenge testing in pool v non-pool athletes. 10% positive test cut off indicated.

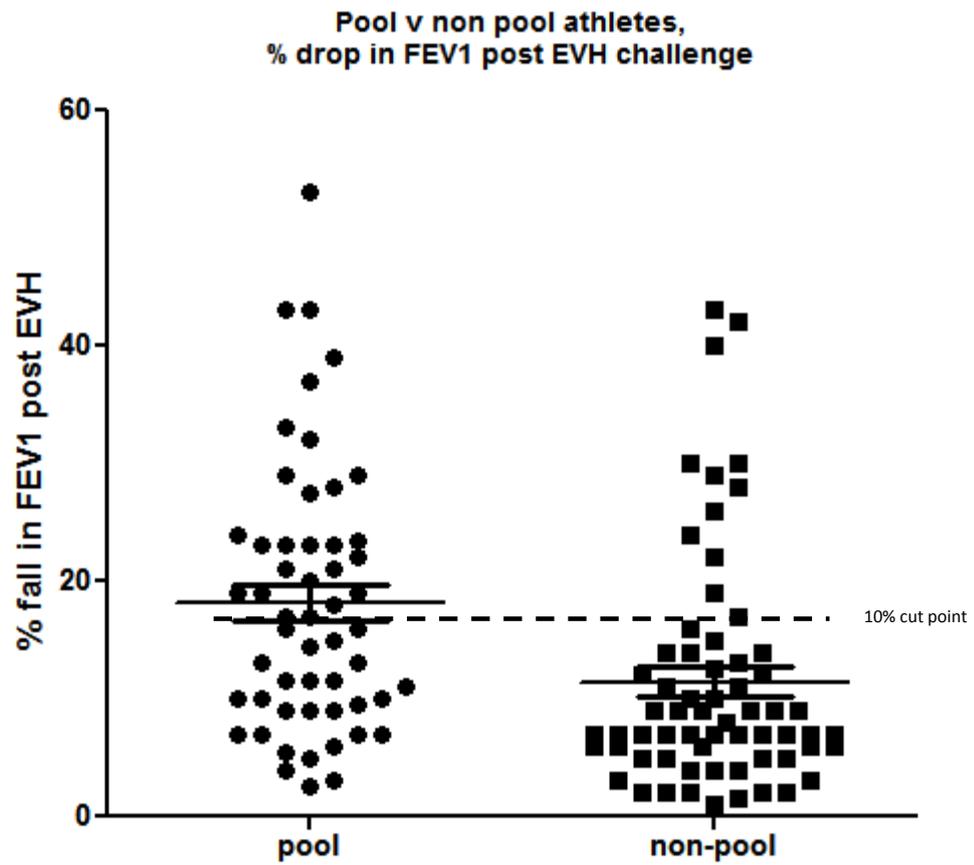


Fig 2: Comparison of asthma symptoms (ACQ mean of 5 item, seven point scale), airways inflammation (log sputum eosinophil %) and response to the EVH challenge test.

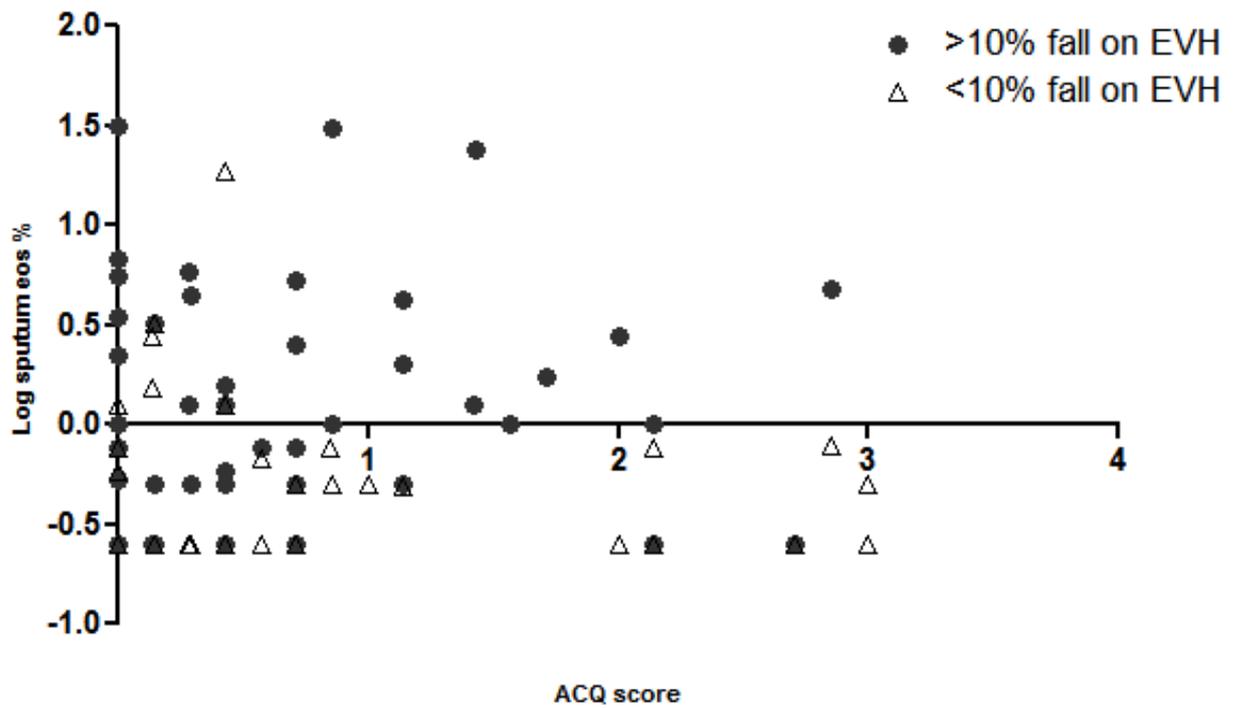
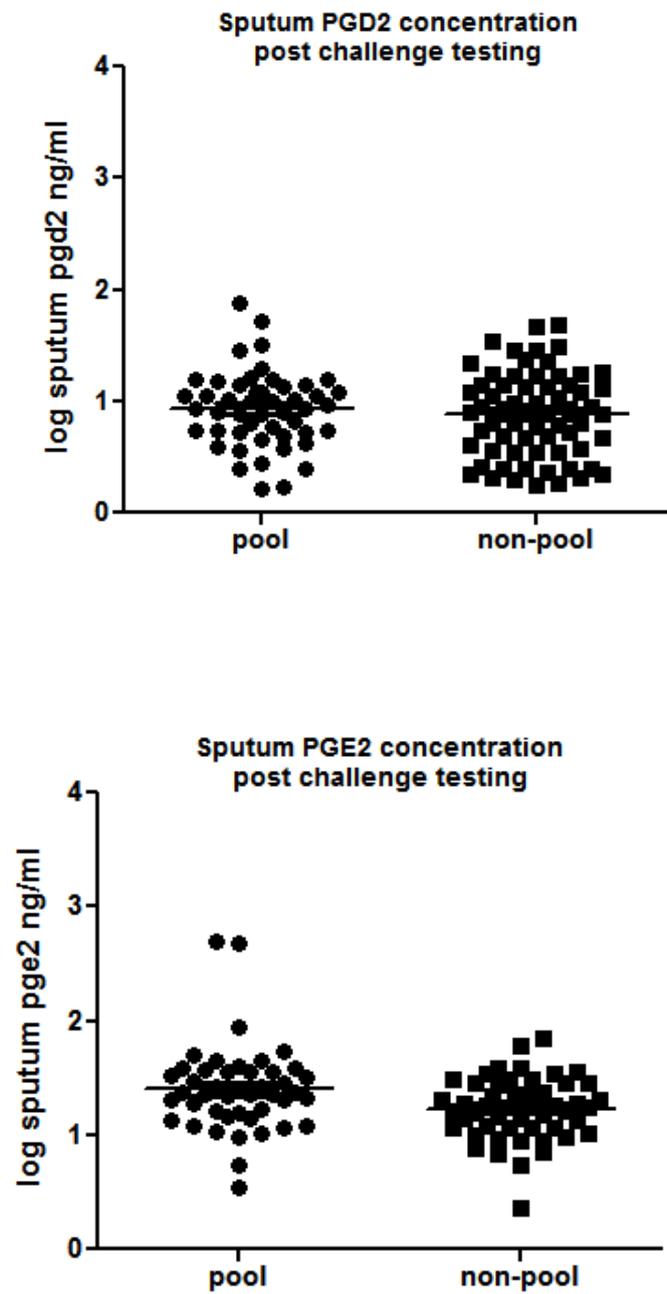
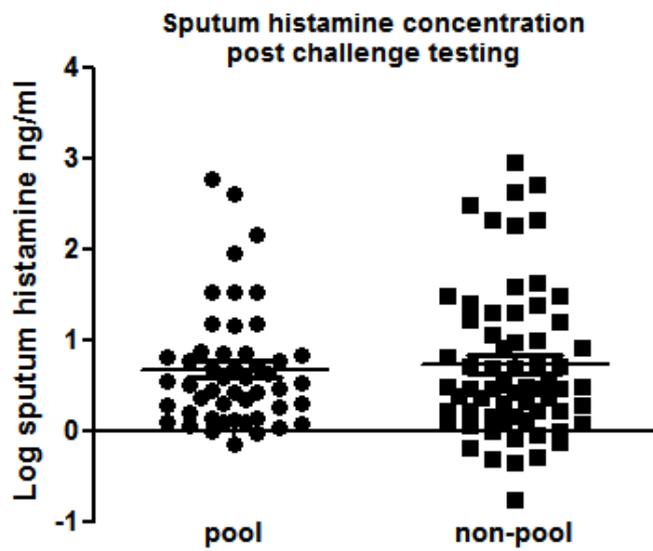
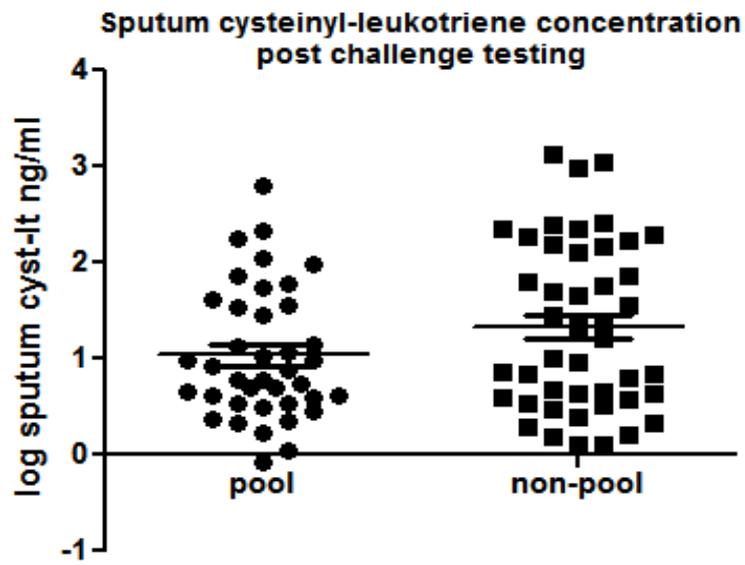


Fig 3. Sputum and urinary mediator concentrations measured post EVH challenge testing in pool and non-pool based elite performance athletes.





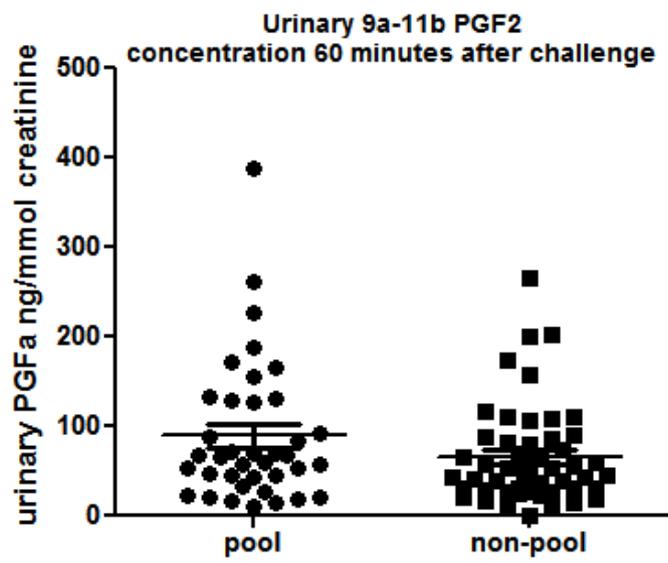
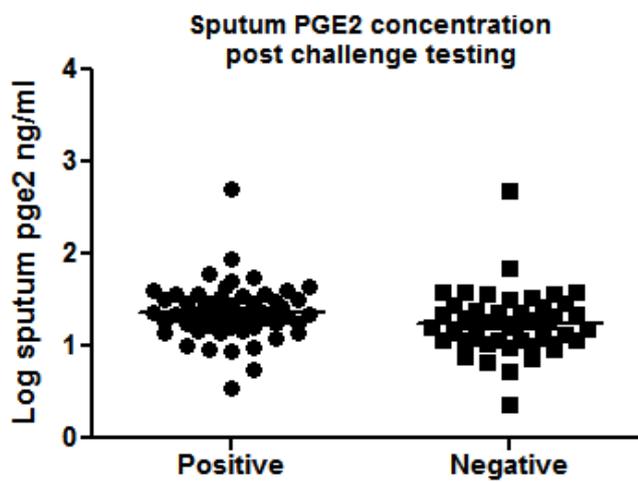
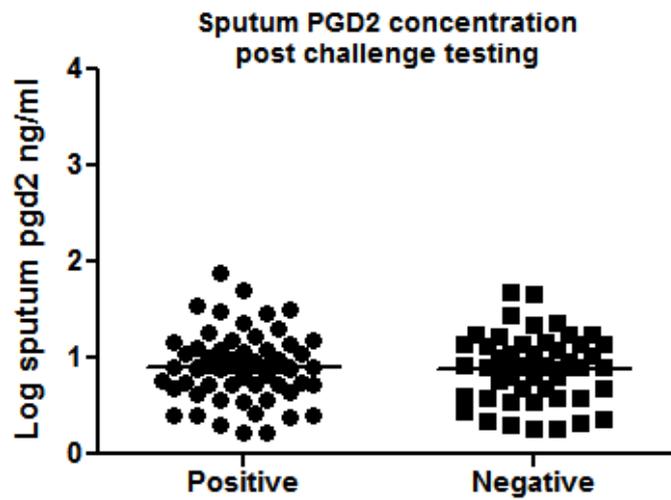
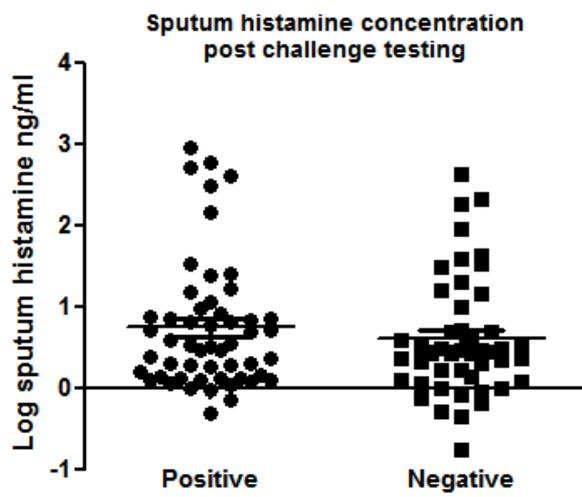
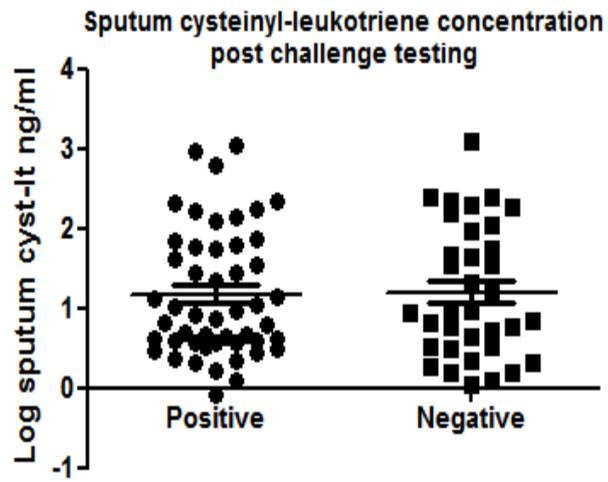
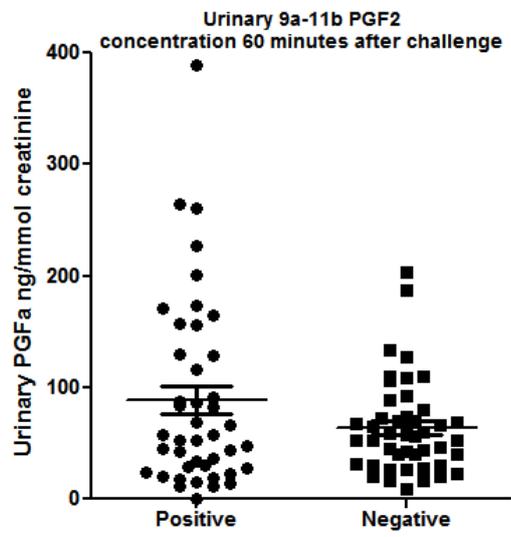


Fig 4. Sputum and urinary mediator concentrations comparing those athletes with a >10% fall in FEV₁ post EVH (positive) with those without (negative) test.







4.3 A soluble mediator acting through a Gi-coupled receptor mediates primary airway epithelial cell-dependent inhibition of human lung mast cell degranulation

Abstract

Chronic mast cell activation is a characteristic feature of asthma. BEAS-2B human airway epithelial cells (AEC) profoundly inhibit both constitutive and IgE-dependent human lung mast cell (HLMC) histamine release. The aim of this study was to examine the regulation of HLMC degranulation by primary AEC from healthy and asthmatic subjects, and investigate further the inhibitory mechanism.

HLMC were co-cultured with both BEAS-2B and primary AEC grown as monolayers or air-liquid interface (ALI) cultures.

Both constitutive and IgE-dependent HLMC histamine release were attenuated by BEAS-2B, primary AEC monolayers and ALI cultures. This occurred in the absence of HLMC-AEC contact indicating the presence of a soluble factor. Unlike healthy ALI AEC, asthmatic ALI-AEC did not significantly reduce constitutive histamine release. AEC inhibitory activity was transferable in primary AEC monolayer supernatant, but less active than with Transwell co-culture, suggesting that the inhibitory factor was labile. The AEC inhibitory effects were attenuated by both AEC wounding and pertussis toxin, indicating the involvement of a G_0/G_i receptor coupled mechanism. Solid phase extraction of lipids (<10 kDa) removed the AEC inhibitory activity. The lipid derivatives resolvin D1 and D2 and lipoxin A_4 attenuated HLMC histamine release in a dose-dependent fashion but were not detectable in co-culture supernatants.

Primary AEC suppress HLMC constitutive and IgE-dependent histamine secretion through the release of a soluble, labile lipid mediator(s) that signals through the G_0/G_i receptor

coupled mechanism. Manipulation of this interaction may have a significant therapeutic role in asthma.

Introduction

Chronic mast cell activation is a characteristic feature of asthma (142;143). There is ongoing production and release of mast cell-derived autacoid mediators and cytokines (144) and morphological evidence of degranulation within asthmatic airways (145). Mast cells infiltrate three key structures in asthma: the airway epithelium (146), the airway submucosal glands (147), and the airway smooth muscle (37). Recent work has highlighted important bi-directional interactions between human lung mast cells (HLMC) and airway smooth muscle, including the ability of ASM to increase constitutive mast cell degranulation (148;149). These interactions are likely to promote ASM dysfunction in asthma.

The outcome of mast cells interacting with the airway epithelium is poorly understood. Airway epithelial cells (AEC) are capable of suppressing mast cell chymase expression (146;150), and supporting mast cell survival (150), in part through the generation of the essential mast cell growth factor, stem cell factor. AEC activated with various stimuli produce TSLP which may induce IL-13 release from cultured mast cells derived from peripheral blood progenitors (151), and mast cells are required for epithelial TSLP expression in a model of allergic rhinitis (152).

We have previously demonstrated that HLMC in contact with BEAS-2B AEC exhibit a marked reduction in both constitutive and IgE-dependent HLMC histamine release (57). Since the airway epithelium in asthma is denuded and expresses an inflammatory phenotype with impaired repair responses (153), we proposed the following hypothesis: that the role of

the healthy intact epithelium is to keep mast cells in a quiescent state, and that tissue insults such as those caused by infection or that present in asthma lead to epithelial damage and denudation which consequently leads to the loss of this bronchoprotective function. If true, this may be critically important in the development of airways hyperreactivity, variable airflow obstruction and airway remodelling.

To further our understanding of the mechanisms regulating HLMC function by AEC, we have now studied the effects of primary human AEC including air liquid interface (ALI) cultures, derived from both healthy and asthmatic subject cultures, on HLMC degranulation.

Methods

BEAS-2B cell culture

The BEAS-2B epithelial cell line culture was carried out as described in the methods section.

HLMC purification and culture, Air-Liquid interface cultures, Mast cell activation, Mediator assays, , Fractionation of AEC supernatants and co-culture supernatants, and Measurement of resolving mediators using liquid chromatography (LC)-mass spectrometry.

These were all carried out as described in the methods section.

Results

A soluble factor(s) mediates BEAS-2B-dependent inhibition of constitutive and IgE-dependent HLMC degranulation

Initially we extended our original work examining the suppression of HLMC degranulation in direct contact with confluent BEAS-2B cells, by comparing the co-culture of HLMC and BEAS-2B cells separated by a 0.40 μm Transwell membrane (BEAS-2B on the underside, HLMC in the top chamber). Analysis of cell supernatants revealed a 55 % (95% CI: 35% - 76%; $p=0.03$) suppression of constitutive histamine release over 16 h in the presence of BEAS-2B in either direct contact (56%; 95% CI: 35%, 76%; $p=0.03$) or separated by a Transwell membrane (54%; 95% CI: 50%, 85%; $p=0.03$) when compared to HLMC cultured alone (Fig.1). There was also a pronounced suppression of IgE-dependent histamine release in the presence of BEAS-2B cells (79%, 95% CI: 62%, 92%; $p=0.03$), which was more pronounced in the presence of the Transwell membrane (89%, 95% CI: 66%, 99%; $p=0.02$) (Fig.1). Similar results were seen when BEAS-2B were present on the top of a Transwell membrane with HLMC in the bottom chamber ($n=7$ HLMC donors) (Fig.2). This was associated with an increase in cellular histamine content in HLMC co-cultured with BEAS-2B Transwells compared to HLMC cultured alone ($n=7$) (Fig.2). BEAS-2B airway epithelial cells therefore consistently inhibit HLMC constitutive and IgE-dependent histamine release, but cell-cell contact is not required indicating that a soluble mediator(s) is involved.

Airway liquid interface cultures of primary airway epithelial cells inhibit HLMC degranulation

We then repeated the latter Transwell experiments described above using ALI AEC cultures that had been grown from well characterised donors either with or without a diagnosis of

asthma. Constitutive (16 h) histamine release was significantly lower from HLMC co-cultured with healthy ALI AEC compared to HLMC co-cultured with asthmatic ALI AEC ($p=0.03$) or HLMC cultured alone ($p=0.01$) ($n=6$ healthy AEC donors, 6 asthmatic AEC donors and 6 HLMC donors)(Fig.3). Asthmatic ALI AEC did not significantly reduce constitutive histamine release compared to HLMC cultured alone ($p=0.07$) (Fig.3). The presence of ALI AEC also significantly suppressed IgE-dependent histamine secretion compared to HLMC monoculture ($p=0.002$) and healthy and asthmatic ALI AEC were equally effective in this respect.

AEC inhibitory activity is transferable in primary AEC monolayer supernatant

To assess further, whether the AEC-inhibitory activity is transferable, we measured constitutive histamine release from HLMC cultured in primary AEC monolayer supernatants for 16 h, compared to control AEC media. There was a significant reduction in constitutive release (shown later in Fig.6), but this was less than that seen in Transwell co-culture, suggesting that the inhibitory factor is labile.

AEC wounding attenuates AEC-dependent HLMC inhibition

To investigate further the mechanism(s) surrounding the inhibitory effect of AEC on HLMC, firstly we wounded a BEAS-2B Transwell monolayer subtly by scraping the monolayer twice with pipette tip to create an X of damaged cells. Interestingly, this manoeuvre markedly reduced the inhibition of the 16 h constitutive histamine release from HLMC ($p=0.03$), but had a much lesser effect on IgE-dependent histamine release ($p=0.29$) (Fig.4) ($n=4$ HLMC donors). This might be in part because wound healing over the 16 h restored some inhibitory effect that was active when the HLMC were subsequently activated with anti-IgE.

EP₂ receptor blockade does not abrogate AEC-dependent HLMC inhibition

PGE₂ is a candidate molecule released by AEC which is known to inhibit HLMC IgE-dependent mediator release both in vitro and in vivo ((157;158)), via the Gs-coupled EP₂ receptor ((159;160)). However, in our previous work, the cyclo-oxygenase inhibitors indomethacin and naproxen had no effect on AEC-dependent HLMC inhibition. In keeping with those observations, a dual EP1/EP2 receptor blocker (AH6809) was without effect, firmly excluding PGE₂ as a mechanism (Fig.4).

Pertussis toxin attenuates AEC-dependent HLMC inhibition

Several anti-inflammatory lipid mediators such as PGE₂, and resolving mediators of inflammation such as lipoxins, resolvins, and protectins operate through Gi-coupled receptors ((161)). We therefore incubated HLMC with pertussis toxin (500ng/ml) overnight in the presence of BEAS-2B AEC Transwell monolayers. This resulted in the complete abrogation of the AEC-dependent inhibition of constitutive histamine release and marked attenuation of the AEC-dependent inhibition of IgE-dependent degranulation (n=4 HLMC donors) (Fig.4).

Resolvin D1 and D2 and lipoxin A₄ attenuate HLMC histamine release

Resolving mediators of inflammation are currently considered to be produced with a delay of several hours following tissue damage, but it is plausible that there is some basal “tone” in this system promoting tissue quiescence. Importantly, these molecules operate through Gi-coupled receptors. Interestingly, preincubation of HLMC with resolvin D1, D2, or lipoxin A₄ for 10 minutes, produced a marked dose-dependent inhibition of subsequent IgE-dependent HLMC histamine release in the dose range 10⁻¹² to 10⁻⁶ M (Fig 6). Even at 10⁻¹² M, HLMC histamine release was reduced by about 50% compared to vehicle control with each of these mediators.

Measurement of resolving mediators in culture supernatants

A commercial ELISA was used to measure lipoxin A₄ in the co-culture supernatants. The lower limit of detection was 0.47 pg/ml but no lipoxin A₄ was detectable. No commercial assays are available for the resolvin family. To assess further whether a related small lipid mediator may be involved, we fractionated AEC supernatants from primary AEC monolayers by removing lipid mediators using reversed phase C¹⁸ solid phase extraction (SPE)(Strata-X, 33 μm, 85 Å, <10 kDa). Removal of lipid mediators ablated the inhibitory activity present in these AEC supernatants (Fig.6). As we were able to transfer inhibitory activity in primary AEC monolayer supernatants, we used liquid chromatography (LC)-mass spectrometry (MS) to look for the presence of resolving mediators (lipoxins, resolvins, protectins, maresins) in both primary AEC monoculture supernatants and AEC-HLMC-co-culture supernatants. We could accurately and reproducibly measure lipoxin A₄ and resolvins D1 and D2 with a lower detection limit of 1 pg/μl (2.7 nM/L) for resolvin D1, and 0.5 pg/μl (1.3 nM/L) for resolvin D2 and lipoxin A₄ (Fig.7). This compares favourably with the detection limit of 11 nM/L for resolvin D1 described by others (¹⁶²). Although we could reliably detect lipoxin A₄, resolvin D1 and D2 in spiked AEC media and spiked supernatants, we were unable to identify lipoxin A₄, resolvin D1 or D2 in AEC/AEC-HLMC co-culture supernatants (n=12). We could also not detect other resolving mediators including resolvin E1 and E2, protectin D1 and maresin 1 which were screened for using published SRM characteristics as listed in Methods.

Discussion

We demonstrated previously that BEAS-2B AEC exert a strong suppressive effect on both constitutive and IgE-dependent HLMC degranulation (57). Suppressive activity in these early

experiments was not significant in freeze-thawed culture supernatants. In this study we have extended our work looking at this interaction between HLMC and human AEC, both in the form of the cell line BEAS-2B and in primary AEC ALI cultures and undifferentiated primary monolayers. We have shown that the inhibition of HLMC degranulation by BEAS-2B is also seen with primary human ALI AEC, and that direct cell contact is not required for this effect. Interestingly, this activity can be transferred, although less efficiently, in the supernatant of primary AEC monolayers, suggesting the presence of a soluble, labile mediator(s).

This mediator is not PGE₂, but signals through a G₀/Gi protein coupled receptor mechanism as evidenced by the reversal of this inhibition in the presence of pertussis toxin. We suggest that good candidate molecules for this AE-derived suppressive effect are the family of resolving mediators of inflammation which include the lipoxins, resolvins and protectins (163). Several lines of evidence support involvement of this class of mediators: i) they have already been shown to be important in epithelial cell function and repair (164) and to have a potential role in airway hyperresponsiveness (165) and asthma (166); ii) they work through G₀/Gi protein coupled receptors (167); iii) their synthesis often requires a contribution from AEC (168); iv) we have demonstrated for the first time that lipoxin A₄, resolvin D₁ and resolvin D₂, are potent inhibitors of HLMC degranulation and v) biochemical fractionation indicates that the activity in AEC supernatant is a <10kDa lipid which would fit the profile of a resolving mediator.

The resolving mediators we tested exhibited a marked inhibition of HLMC degranulation at concentrations well below the current detection limits of current lipoxin ELISAs (approximately 10⁻¹⁰ M) or high performance mass spectrometry techniques (approximately

10^{-8} M (162). Measuring these in their biologically active range is therefore challenging. We were unable to detect lipoxin A₄, resolvin D1 or resolvin D2 using LC-MS although spiked standards were detected in culture media, and could not detect Lipoxin A₄ by ELISA. We could also not detect other resolving mediators (resolvin E1 and E2, protectin D1 and maresin 1) by LC-MS which were screened for using published SRM characteristics. However, such molecules could well be present and exert significant biological activity well below the currently available levels of detection. As well as the characterised resolving mediators mentioned above, there are also potentially hundreds of other arachidonic acid, eicosapentanoic acid and docosahexanoic acid metabolites which might exert similar effects (169).

Three further independent studies support our findings (150;170;171). Mast cells freshly isolated from human nasal polyp epithelium released only 2% of their histamine following IgE-dependent activation compared to 22% from cells freshly isolated from the polyp stroma. In contrast, both populations of mast cells released ~50% of their total histamine with calcium ionophore. Hsieh and colleagues demonstrated that primary AEC cultures maintain the survival of human cord blood-derived mast cells, and although not discussed, reduced histamine, PGD₂ and LTC₄ release compared to mast cells alone by ~50%. Furthermore, rat basophilic leukaemia cell degranulation was inhibited by a soluble mediator of <3kDa present in the supernatant of BEAS-2B (171).

In the current work we have investigated the effect of primary AEC derived from both healthy and asthmatic subjects. ALI cultures derived from healthy subjects behaved in a very similar fashion to BEAS-2B, exhibiting a strong suppressive effect on both constitutive and IgE-dependent HLMC histamine release. Interestingly, ALI cultures from asthmatic

individuals were less effective at suppressing constitutive HLMC histamine release, but were still very effective at inhibiting IgE-dependent release. This raises the possibility that there is an intrinsic defect in asthmatic AEC which results in loss of the brake on background mast cell activity, which may therefore predispose to the development of atopy and asthma.

Alternatively there may be two mediators active in this system, one which targets constitutive mast cell mediator release and which is relatively deficient in asthma and another which targets IgE-dependent release.

The signalling pathways involved in IgE-dependent mast cell degranulation are known in some detail (150;170). However, little is known about the factors influencing constitutive mast cell mediator release. Our data show clearly that this can be modulated. IgE-dependent activation results in the fusion of granules and the formation of large degranulation channels, so-called anaphylactic degranulation (172). While this appearance can be seen in asthmatic airways, the typical mast cell morphology here and in other disease tissues is that of piecemeal degranulation, where the granule membranes remain intact with variable loss of granule contents (173). Whether this is an extension of the constitutive release evident *in vitro*, or yet another release pathway is not known. If the former, then the loss of AEC inhibitory activity in asthmatic airways could explain the morphological appearance of mast cells in asthma, and the chronically elevated concentrations of mast cell-derived mediators identified previously (174). The impaired inhibition of constitutive HLMC histamine release by asthmatic AEC and the further loss of inhibition following AEC wounding, may therefore be a fundamental abnormality which contributes to the development and propagation of asthma.

It is noteworthy that subtle damage to the AEC markedly attenuated the inhibitory effect, most evident when looking at constitutive HLMC histamine release, but also with IgE-dependent release. This suggests a dynamic process was operative, rather than mechanical loss of AEC being responsible. Identifying the inhibitory mediator(s) and understanding the AEC signalling pathways behind this are important future goals.

In summary we have demonstrated that primary asthmatic AEC are less effective at suppressing the constitutive release of histamine from HLMC than healthy AEC, while both attenuate IgE-dependent degranulation. These suppressive effects are attenuated by AEC wounding, and by disabling $G_{0/i}$ -coupled receptors. A soluble lipid mediator(s) is involved, and the family of lipid-derived resolving mediators are candidates, supported by the observation that lipoxin A4, resolvin D1 and resolvin D2 are potent inhibitors of HLMC IgE-dependent activation. These mediators may therefore have potential use as mast cell-targeted therapies. Identifying the inhibitory mediator(s) derived from AEC and understanding the AEC signalling pathways behind its production offers exciting potential for the development of novel approaches to treating asthma.

Fig 1. Constitutive (16 h) and IgE-dependent (30 min) HLMC histamine secretion in Monoculture and in the presence of BEAS-2B cells, either in contact or on either side of a Transwell membrane (BEAS-2B on the underside of the membrane, HLMC on the upperside of the membrane) . Mean \pm SEM ng/10⁶ cells (N=3 HLMC donors, *p=0.03, **p=0.02)

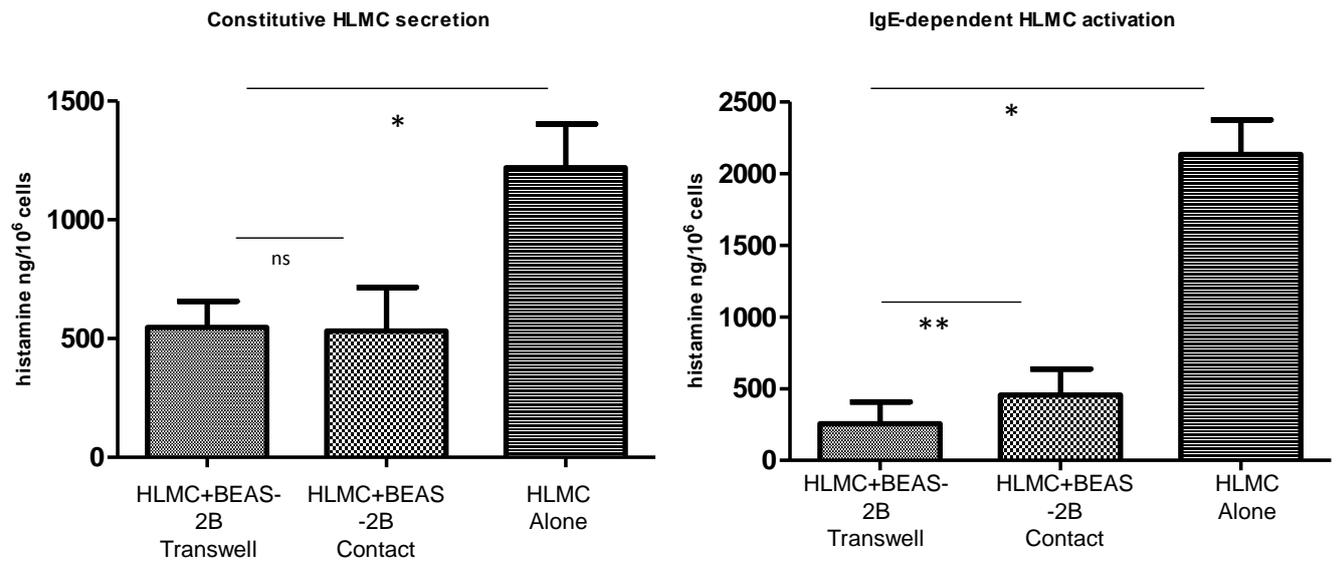


Fig 2. Constitutive (16 h) and IgE-dependent (30 min) HLMC histamine secretion in monoculture or in the presence of BEAS-2B cells separated by a Transwell membrane (HLMC in the bottom chamber, BEAS-2B in the top chamber). Retained cell histamine content prior to activation is also shown. n=7 HLMC donors. Mean \pm SEM ng/10⁶ cells.

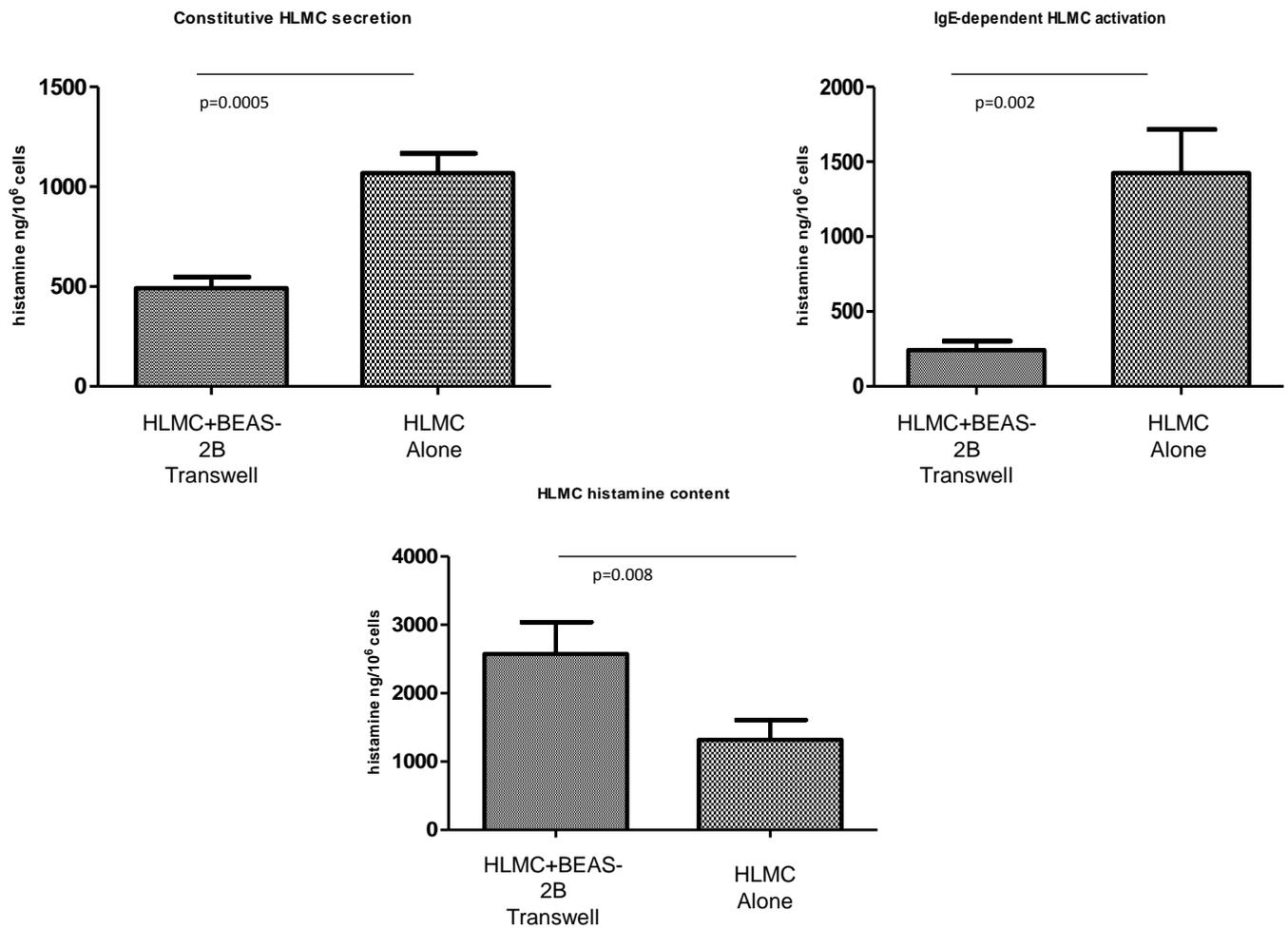


Fig 3. The effects of HLMC co-culture with healthy and asthmatic ALI AEC on constitutive (16 h) and IgE-dependent (30 min) HLMC histamine secretion compared to HLMC cultured alone, n=6 HLMC donors. Mean \pm SEM ng/10⁶ cells.

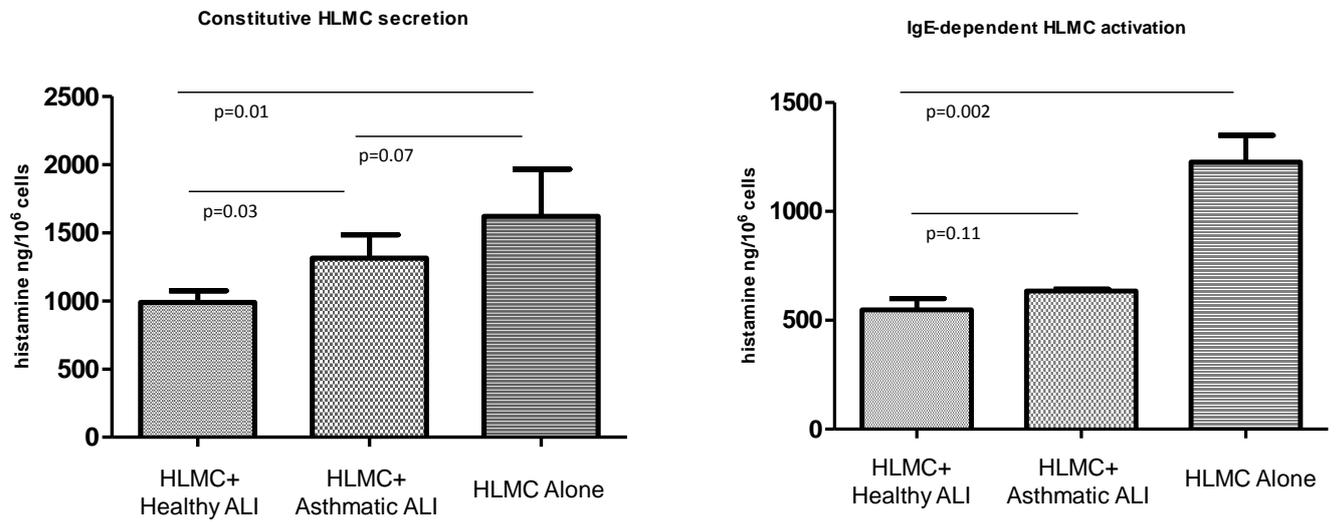


Fig 4. The effects of AH6809, epithelial wounding and pertussis toxin on epithelial-dependent inhibition of HLMC histamine secretion n=4 HLMC donors, mean \pm SEM ng/10⁶ cells.

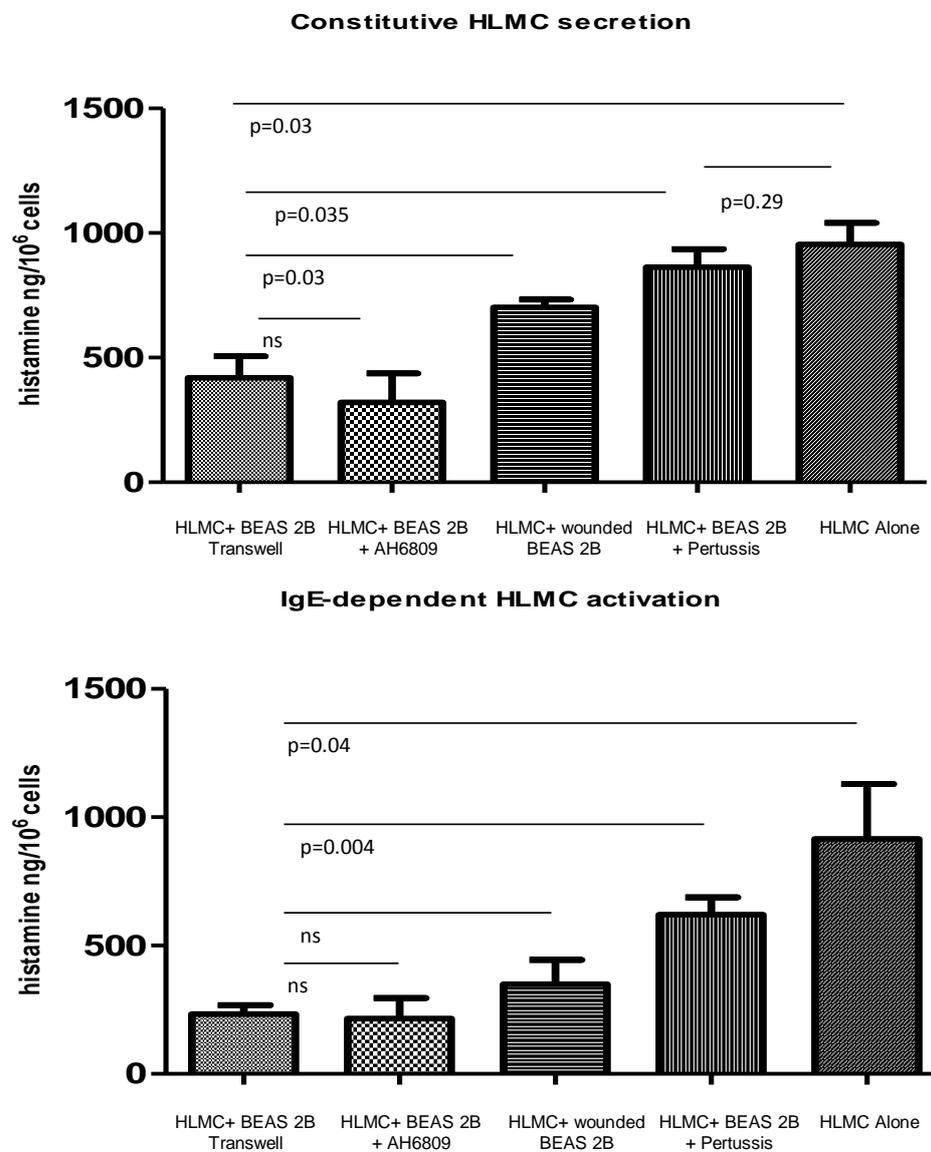


Fig 5. The effects of lipoxin A4, resolvin D1 and resolvin D2 on HLMC IgE-dependent degranulation. Mean \pm SEM from 7 HLMC donors. ($p < 0.001$ for all mediators (repeated measures ANOVA)).

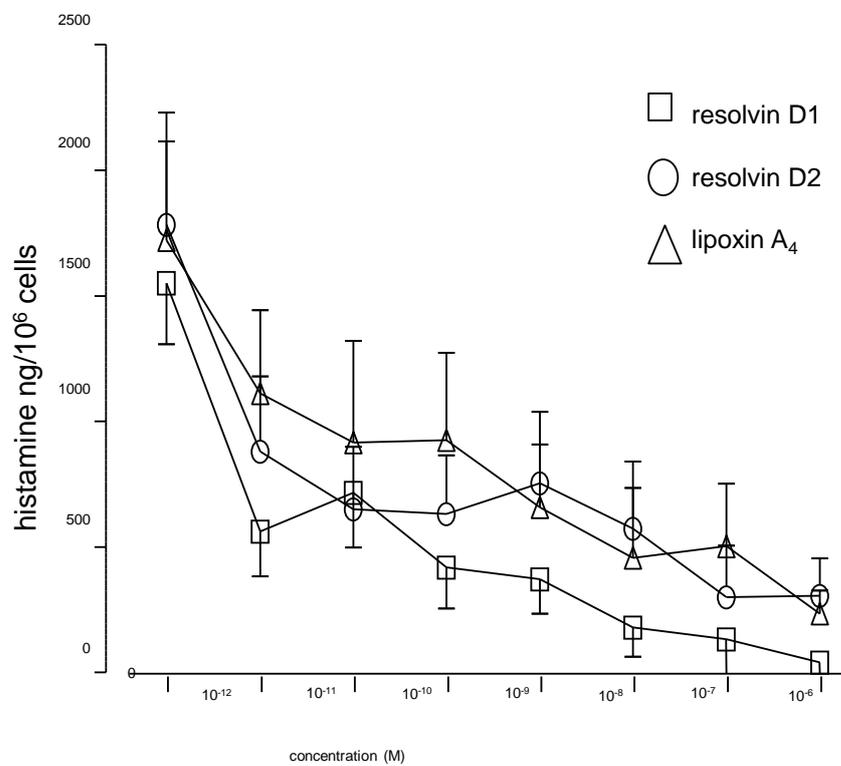


Fig 6. HLMC histamine release in different culture media: E – primary AEC monolayer supernatant; E-SPE – SPE-processed (lipid-deplete) AEC supernatant; BEGM-SPE – SPE processed BEGM media; BEGM – unprocessed media. Mean \pm SEM of 4 experiments using 2 AEC donors and 2 HLMC donors. HLMCs cultured in AEC supernatant showed suppressed histamine release ($p < 0.05$) whereas there is no significant suppression in lipid-deplete supernatant.

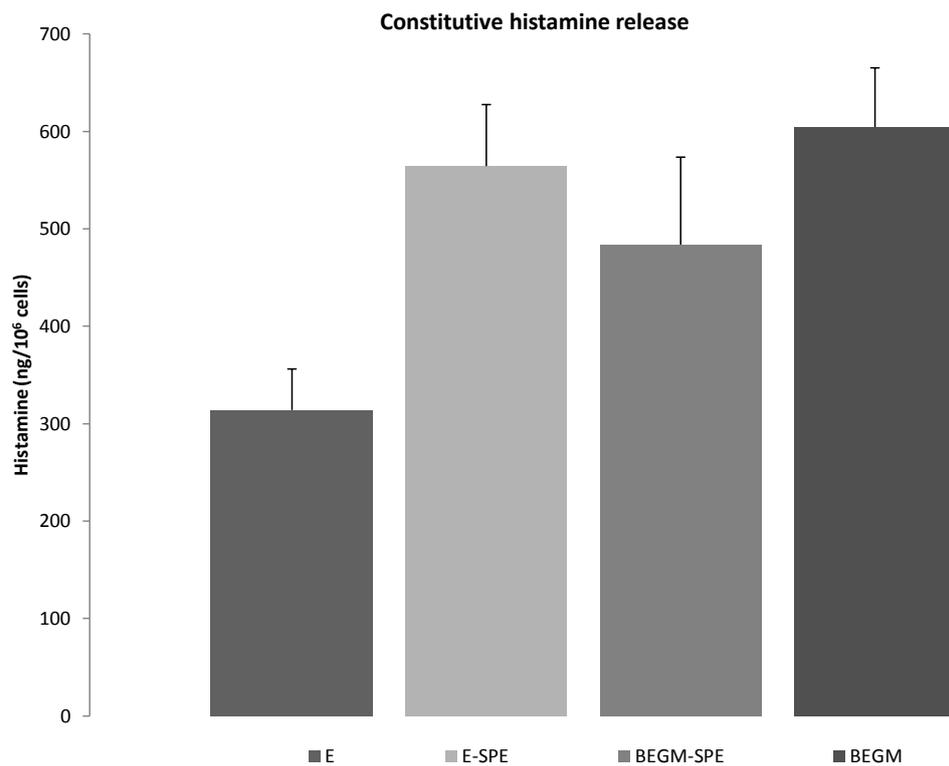
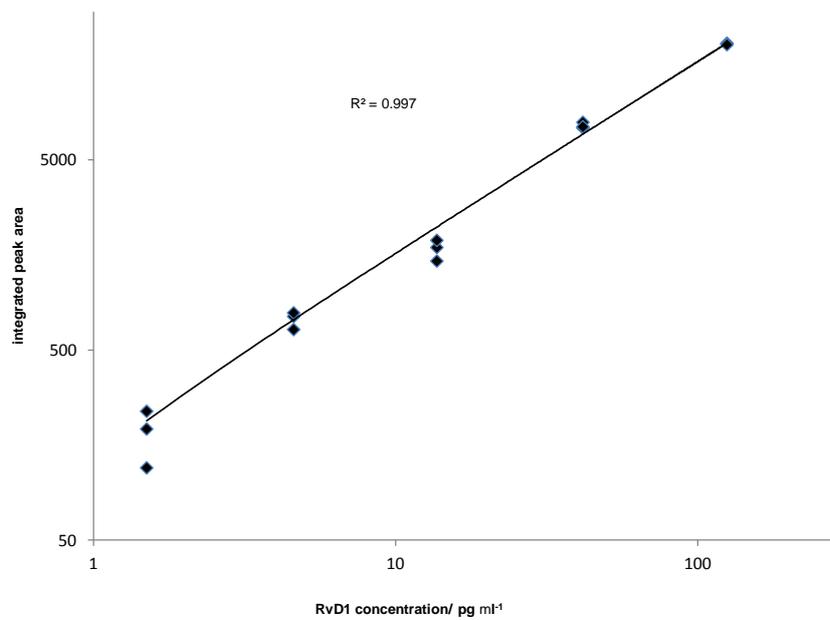


Fig 7. LC-MS calibration curve for triplicate injections of resolvin D1 standard with a linear regression best fit line. Integrated peak area refers to the 375.2 → 141.2 ion pair.



5.0 Conclusions:

5.1 Summary of findings

Asthma and exercise induced bronchoconstriction remains a major issue in elite level sport, with quite marked effects on athlete performance and health. There is no specific phenotype of airways disease based on athlete, sport, type of sport, or sporting environment. Rather, asthma in elite athletes is a heterogeneous disease with varying levels of eosinophilic airways inflammation and airways hyperreactivity.

Symptom expressions and symptom scores are unhelpful in delineating those who have either eosinophilic airways inflammation or airways hyperreactivity. Athletes with a prior history of atopy or childhood disease are much more likely to have positive responses to challenge tests and to have significant eosinophilic airways inflammation and this can easily and effectively be assessed non-invasively by measuring the fraction of exhaled nitric oxide as a 'pitchside' test. Swimmers (or pool based) athletes are more likely to have airways hyperreactivity but are no more likely to have eosinophilic airways inflammation.

Testing for exercise induced bronchoconstriction in athletes also provides its' challenges. There are heterogenous responses to indirect challenge tests amongst athletes with similar symptom histories and airways inflammatory patterns. Eosinophilic patients are much more likely to have a positive test result and in this respect both methacholine and the EVH test performed well. Of particular note was the increased number of epithelial cells in the sputum of those with a positive test, suggesting that epithelial desquamation or injury may have a role to play in the development of airways hyper-responsiveness in athletes.

Finally, in vitro work has shown a clear relationship between a healthy, intact epithelium and the suppression of mast cell constitutive histamine release. This interaction is through a soluble, labile, lipid mediator that may be from the resolving family of pro-resolution compounds. Further exploitation of this mechanism has exciting therapeutic potential.

5.2 Criticisms

We did not find much evidence that mast cell mediators were more involved in the response to indirect tests when compared to direct tests both in the sputum supernatant and the urine. A baseline sample from sputum would have required another visit (on top of the 4 carried out) for the athletes and was impractical because of their training schedules and commitments. A pre- and post-test urine specimen would have been possible but we felt that there would have been significant differences between the direct and indirect tests which would have acted as a bronchoconstriction control. It would also have been good to assess the athletes' response to inhaled corticosteroids. Indeed this was attempted but the follow up visits were so badly attended that the data was of little use and a follow up questionnaire received a very low response rate, and generally replies were best from amongst those who had a good response to steroids. This demonstrates the problems encountered when doing research in such a specialized, high achieving and otherwise intensely focused group of individuals.

It would also have been good to determine exactly the contribution of the resolving family of mediators to mast-epithelial cell interaction. However, this is still an important finding and there is much work still being done to refine this hypothesis and delineate options for manipulating the interaction. Other groups have also been working on similar models, with similar results.

5.3 Future work

As always, this research has perhaps raised more questions than it has answered. From the clinical perspective it has strongly suggested that symptoms alone are insufficient for making a diagnosis of asthma in elite level athletes. They require a thorough assessment in terms of evidence of airways inflammation, airways hyper-responsiveness and evidence of alternative diagnoses such as vocal cord dysfunction and dysfunctional breathing. The reduction in scrutiny for elite athletes using asthma medication in sport will undoubtedly lead to a reduction in challenge testing of athletes and a reversal to symptom driven prescribing and this will lead once again to inappropriate use of medication at this level. Mostly this will be driven by the cost of testing and the establishment of an NHS clinic providing athletes access to such in depth analysis for performance may aid with continuing a high degree of scrutiny in this field.

Our cell culture interactive model requires further exploitation. Whilst we know that the mediator(s) is a small, soluble, labile, lipid mediator that acts through a Go/Gi protein coupled receptor complex we have not been able to definitively show that this is a resolvin. Further delineation of this pathway is important for several reasons. It might help explain why diets rich in omega-3 fish oils are protective for childhood asthma, why some athletes 'asthma' improves with fish oil supplementation and more basically how airways hyper-responsiveness develops. If we can boost this endogenous protective pathway in children perhaps the epidemic of asthma we are currently seeing is correctable to some degree. It would be particularly useful if this could be done with natural supplementation rather than pharmaceutical means but the exploitation of this pathway may also lead to enhanced therapeutic options in established disease.

Finally, there has been a resurgence in interest in the role of the epithelial cell in lung disease as a whole, but in asthma particularly. The epithelial cell may have a more important position than previously thought in the development of airways hyper-responsiveness and the development of airways inflammatory change. The response to external challenges such as viruses and toxins seems central to this and these processes require further elucidation. Much more work needs to be carried out in this field and in particular examining the changing epithelial phenotypes seen in young children who are at risk of developing asthma may yield further insights into the loss of this protective mechanism and the subsequent development of asthma.

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